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NOVEL INSIGHTS IN THE SERODIAGNOSIS AND PATHOGENESIS OF SWINE INFLUENZA

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List of abbreviations

Abs	antibodies
APP	acute phase proteins
BAL	broncho-alveolar lavage
BALF	broncho-alveolar lavage fluid
BP	binding protein
CDCD	caesarean-derived colostrum-deprived
CPE	cytopathogenic effect
CRP	C-reactive protein
EID_{50}	mean egg infectious dose
ELISA	enzyme-linked immunosorbent assay
HA	hemagglutinin
HG	haptoglobin
HI	hemagglutination inhibition
HSV-1	herpes simplex virus type 1
ICE	IL-1β converting enzyme
IFN	interferon
Ig	immunoglobulin
IFNAR	IFN-α receptor
IL	interleukin
IP	intraperitoneal
IP-10	IFN-inducible protein 10
IPMA	immunoperoxidase monolayer assay
IRF	IFN regulatory factor
ISRE	IFN-stimulated response element
IT	intratracheal
IV	intravenous
JAK1	janus kinase 1
LBP	LPS binding protein
LPS	lipopolysaccharide
MDBK	Madin-Darby bovine kidney
MDCK	Madin-Darby canine kidney
MIG	monokine induced by IFN-γ
Mx	orthomyxovirus resistance
NA	neuraminidase
NF-κB	nuclear factor κ B
NLF	nasal lavage fluid
NP	nucleoprotein
NPV	negative predictive value
NS	non-structural protein
NU	neutralizing units
OAS	2',5'-oligoadenylate synthetase
OD	optical density
P	probability
PBS	phosphate-buffered saline
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PI	post inoculation

PK	porcine kidney
PKR	protein kinase R
PPV	positive predictive value
PRCV	porcine respiratory coronavirus
PRV	pseudorabies virus
ρ	Spearman rank correlation coefficient
RBC	red blood cells
SD	standard deviation
SEM	standard error of the mean
SIV	swine influenza virus
STAT	signal transducer and activator of transcription
S/P	sample to positive
$TCID_{50}$	mean tissue culture infectious dose
TNF	tumour necrosis factor
TYK2	tyrosine kinase 2
U	units
VN	virus neutralization
VSV	vesicular stomatitis virus

1.

INTRODUCTION

1.1 INTRODUCTION ON SWINE INFLUENZA

1.1.1 Brief historic overview of influenza

The Greeks already knew human influenza and a first epidemic was recorded by Hippocrates in 412 B.C. The viruses are now classified in the family of the Orthomyxoviridae, which has a Greek etymology: orthos meaning "standard, correct" and myxo "mucus" (Lamb and Krug, 2001). The name influenza comes from the Italian "influenza delle stelle" because in the Middle Ages people believed that there was an astrological influence on the disease. Several pandemics stroke the world with the "Spanish flu" of 1918 being the most famous (Wright and Webster, 2001). Swine influenza was first recognized as a clinical entity in 1918 and its pathology was described for the first time by Shope in 1931 (Shope, 1931a). The etiology of this disease was first assigned to a bacterium, *Haemophilus influenzae suis* (Lewis and Shope, 1931). Because the intranasal inoculation of bacteria free filtrates of the lungs of influenza-infected pigs resulted in mild disease, a viral etiology of the disease was suggested. A synergistic effect with *H. influenzae suis* was presumed since inoculation of filtrates together with this bacterium resulted in more pronounced symptoms (Shope, 1931b). Today this bacterium is named *H. parasuis*, the causative agent of porcine polyserositis and arthritis or Glässer's disease (Killian, 1976). The viral etiology of human influenza was discovered shortly thereafter (Smith *et al.*, 1933). The virus was first cultured in embryonated chicken eggs (Burnet, 1936). Propagation of influenza viruses in the amniotic cavity of embryonated chicken eggs was at first the method of choice since virus replication was supposed to take place in the embryo itself (Burnet, 1940). Influenza virus propagation in the membranes surrounding the allantoic cavity was demonstrated one year later and turned out to be a more practical technique (Burnet, 1941). This method is still the method of choice for propagation of influenza viruses for e.g. vaccine production. Influenza virus vaccines were developed in the 1940s by the United States military. The first vaccines used crude preparations of infected chick embryo tissues or mouse lungs (Francis, 1953). It is estimated that seasonal influenza in humans causes worldwide about 250 000 to 500 000 deaths each year. About 250 million doses of influenza virus vaccine are marketed each year, requiring hundreds of millions of embryonated chicken eggs (Gerdil, 2003).

1.1.2 Swine influenza virus: nature and evolution

Influenza viruses belong to the family of the Orthyomyxoviridae. The members are negative, single stranded RNA viruses with a segmented genome, an envelope and a diameter of approximately 80-120 nm. There exist 5 genera within the Orthomyxoviridae family: type A influenza virus, type B influenza virus, type C influenza virus, Thogotovirus and Isavirus (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_ortho.htm). Each of these genera is characterized by differences in nucleoprotein (NP) and matrix (M1) proteins which are both internal structural proteins. The M2 protein is a minor envelope glycoprotein that serves as an ion channel that permits ions to enter the virion during uncoating and thus favours genome release. Three RNA-dependent RNA polymerases (PA, PB1 and PB2) are important in the replication of the genome. Amongst the RNA viruses, influenza is exceptional because replication of the genome takes place in the nucleus. Only the non-structural protein 1 (NS1) is expressed during infection while the NS2 is a structural protein that functions as a nuclear export protein. Hemagglutinin (HA) and neuraminidase (NA) are the main envelope glycoproteins of influenza A radiating out of the lipid envelope and they play a role in entry and release of the virus from the infected cell, respectively (Lamb and Krug, 2001). A schematic representation of an influenza virion is given in figure 1.

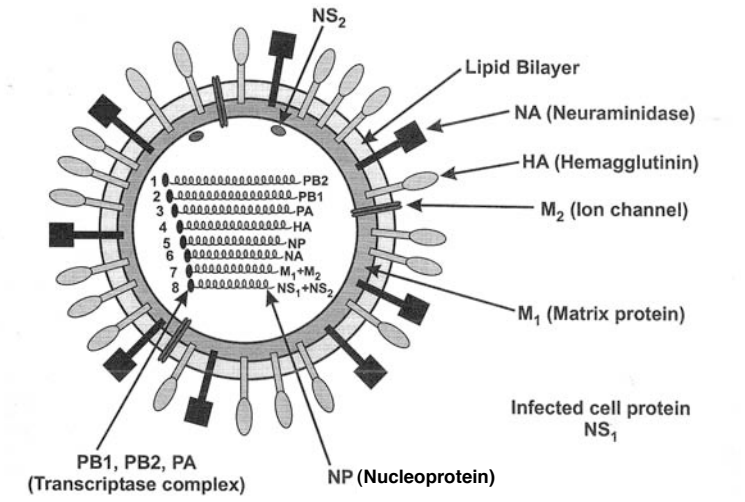

Figure 1. Structure of an influenza viron. The figure shows the different proteins of an influenza virus (adapted from Lamb and Krug, 2001).

HA and NA molecules are used to classify influenza viruses in different subtypes. There are 16 different HA and 9 different NA (Fouchier *et al.*, 2005). These antigenic subtypes are distinguished by double immunodiffusion assays with hyperimmune animal sera as instructed by the World Health Organization (Assaad *et al.*, 1980; Röhm *et al.*, 1996). This classification is reinforced by amino acid sequence comparisons, which indicate 40-80% sequence identity between subtypes and about 90% identity within subtypes (Nobusawa *et al.*, 1991).

Influenza viruses infect a wide variety of avian and mammalian species. In birds all known HA and NA subtypes circulate in at least 82 different combinations (Olsen *et al.*, 2006). Avian influenza viruses can be found predominantly in waterfowl, order *Anseriformes*. *Passeriformes* and *Charadriiformes* are the other two orders of birds from which influenza viruses are mainly isolated (Stallknecht and Shane, 1988). Domestic poultry such as chickens, turkeys, ducks, geese, quails, pheasants, ratites and caged pet birds are also susceptible to influenza viruses. In poultry there are two pathotypes of influenza viruses. The very virulent viruses cause "fowl plague", now termed highly pathogenic influenza (HPAI), and these viruses have a H5 or H7 HA with a polybasic sequence inserted at the cleavage site of the HA. The other viruses cause a much milder disease, designated low pathogenicity avian influenza (LPAI), and can have any HA subtype (Alexander, 1999). In mammals influenza viruses can be found in humans, swine and horses and occasionally in other species such as felidae and marine mammals. The research in this thesis focuses on swine and therefore only this species will be further discussed.

In swine, different influenza A virus subtypes are present. Currently H1N1, H1N2 and H3N2 swine influenza virus (SIV) subtypes are endemic in swine in Belgium (Labarque *et al.*, 2004). The current H1N1 SIV emerged in the European swine population in 1979 and replaced the classical H1N1 viruses (Scholtissek *et al.*, 1983). All genes from the current virus are almost identical to those of a duck H1N1 influenza virus and consequently the H1N1 SIV is called "avian-like" (Brown *et al.*, 1997). The first isolation of a H3N2 influenza virus in pigs was in Taiwan in 1969 and the virus is most closely related to the human A/Hong Kong/68 virus. The virus has been circulating ever since in swine (Ottis *et al.*, 1982) and disease in pigs was first described in 1984. The currently circulating H3N2 SIV originated from a reassortment that took place half way the 1980's, resulting in a virus with internal protein genes from the "avian-like" H1N1 virus and HA and NA genes from a

"human-like" H3N2 influenza virus (Castrucci *et al.*, 1993). The H1N2 virus is the most recent SIV and was isolated for the first time in 1994 in Great Britain from pigs suffering from a respiratory outbreak (Brown *et al.*, 1995). Afterwards it was also isolated in Italy, France, Belgium, Germany and Spain (Van Reeth *et al.*, 2000). It is a "double-reassortant" with HA from a human H1N1 virus from the 1980s, NA from a swine H3N2 virus and internal genes from an "avian-like" swine H1N1 virus (Brown *et al.*, 1998). Although the same SIV subtypes circulate in Europe, Asia and the United States they are antigenically and genetically very different in different continents (Olsen *et al.*, 2006). Besides the above-mentioned SIVs, pigs can also be naturally or experimentally infected with a wide variety of LPAI viruses and thus the pig might play a role in the generation of new pandemic influenza strains (Kida *et al.*, 1994; Karasin *et al.*, 2004; Van Reeth, 2007; Lee *et al.*, 2009). Serologic evidence for highly pathogenic H5N1 avian influenza viruses in pigs was found during the recent epizootics of this virus (Choi *et al.*, 2005). A recombinant human influenza virus with the NS protein from a HPAI virus resulted in a prolonged virus replication, fever and weight loss in pigs. This might in part explain the unusual pathogenicity of HPAI in humans (Seo *et al.*, 2002).

In Belgium and Europe SIVs are very widespread (Maes *et al.*, 1999, 2000; Labarque *et al.*, 2004; Van Reeth *et al.*, 2008). The prevalence of farms with antibodies to swine influenza in different European countries is very heterogeneous as shown in Table 1. The percentage of farms with antibodies to the novel H1N2 SIV is generally lower than the percentage of farms with antibodies to H1N1 or H3N2 SIV. In Belgium, Germany, Italy and Spain the novel H1N2 SIV has become widespread and cocirculates with H1N1 and H3N2 SIV. In the Czech Republic, Ireland and Poland, in contrast, both H1N2 and H3N2 SIV are either undetectable or found at low levels. The dominant SIV subtype in these countries is H1N1 but its prevalence is lower than in the first series of countries. The percentage of influenza seronegative farms ranges from only 1% in Belgium to 91% in Poland. A lower pig density or differences in the structure of the pig industry might in part explain the lower prevalence of swine influenza in some countries (Van Reeth *et al.*, 2008).

Table 1. Percentage of farms with antibodies to H1N1, H3N2 and H1N2 swine influenza virus in seven European countries (adapted from Van Reeth *et al.*, 2008).

country	percentage of farms with antibodies to			percentage of farms without SIV antibodies
	H1N1	H3N2	H1N2	
Belgium	97	86	89	1
Germany	94	91	75	2
Italy	83	68	36	9
Spain	65	67	87	4
Czech Rep.	38	5	16	47
Ireland	42	16	2	44
Poland	9	0	0	91

1.1.3 Swine influenza: pathogenesis, diagnosis and prevention

Up to 50% of the acute outbreaks of respiratory disease in pigs on commercial farms are caused by SIV or by a combination of SIV with bacteria (Loeffen *et al.*, 1999). Upon infection, SIV is generally limited to the respiratory tract. The main target cells are bronchiolar and alveolar epithelial cells. The virus replicates to very high levels in the lungs (up to 10^8 mean tissue culture infectious dose ($TCID_{50}$) per gram of tissue) (Van Reeth *et al.*, 1999; Heinen *et al.*, 2000) and is shed via nasal secretions (Van Gucht *et al.*, 2006). Transmission of SIV occurs through nasal virus shedding, pig-to-pig contact and aerosol formation. The disease spreads rapidly in a herd and virus clearance within an animal takes place within 1 week after infection (Van Reeth, 2007).

Infection with the 3 SIV subtypes results in a clinically undistinguishable disease. The incubation period is 1-3 days and disease onset is sudden. Morbidity is high but mortality is less than 1% in case of proper treatment against secondary bacterial infections. The clinical signs are comparable to those of human influenza and consist of fever (rectal temperature higher than 40°C), anorexia, weight loss, lethargy, rhinitis, nasal discharge, coughing, sneezing and dyspnea. The animals recover within 1 week after onset of the disease (Gillespie, 1999; Heinen *et al.*, 2000; Van Reeth *et al.*, 2001b; Kitikoon *et al.*, 2006; Olsen *et al.*, 2006). This clinical appearance can be observed following both a natural infection and an experimental intratracheal inoculation with a high virus dose. Subclinical influenza virus infections occur

frequently because most pigs are serologically positive for one or more influenza virus subtypes at the end of the fattening period, without observation of clinical symptoms of influenza (Maes *et al.*, 1999, 2000; Olsen *et al.*, 2006).

Macroscopic lung lesions are those typical for a viral catarrhal pneumonia as illustrated in figure 2. Lesions consist of dark-red consolidated zones, which are firm and not collapsible, visible at the dorsal and ventral side of mainly the apical and cardiac lung lobes. The demarcation line between normal and affected lung zones is sharp (Gillespie, 1999; Kitikoon *et al.*, 2006; Kothalawala *et al.*, 2006). This pattern is seen in both natural and experimental infections and might be caused by the fact that the upper lung lobes are easier to reach for the inhaled virus than the lower lung lobes in a natural infection or the inoculum in an experimental infection. Microscopically, there is necrosis of the epithelia, desquamation of the bronchial epithelial cell layer, necrotizing bronchiolitis and bronchointerstitial pneumonia. In early infection stages, there is infiltration of neutrophils in the airways and the bronchioles are plugged with necrotic debris (Gillespie, 1999; Van Reeth *et al.*, 1999; Heinen *et al.*, 2000). A representation of the microscopic lung lesions is given in figure 3. Lymphocytes infiltrate in the lungs a few days later.

Figure 2. Typical macroscopic lung lesions (dark-red consolidated zones) in apical and cardiac lung lobes after intratracheal inoculation of $10^{6.0}$ EID_{50} swine influenza virus (H3N2).

A clinical diagnosis of swine influenza is only presumptive since symptoms of swine influenza and other respiratory diseases resemble to some extent (Olsen *et al.*,

2006). Diagnosis can be done either by virus isolation, detection of viral antigens or serological techniques to demonstrate virus-specific antibodies (Janke, 2000). For virus isolation nasal exudates or homogenates from post-mortem lung samples taken at the acute stage of the infection can be used. Virus isolation can be done either in chicken eggs or in cell cultures followed by a hemagglutination test or a staining for viral antigens, respectively. Isolation necessitates virus that is not inactivated and thus samples must be kept moist and refrigerated. Lungs of influenza infected pigs can be used in an immunofluorescence or immunoperoxidase staining to detect viral antigens which is conclusive for an ongoing influenza infection. Polymerase chain reaction (PCR) tests were also developed for the detection of influenza viruses and the different subtypes can be identified by the use of the subtype specific primers HA1, HA3, NA1 and NA2 (Choi *et al.*, 2002; Lee *et al.*, 2008). The PCR can be performed on a wide range of samples including nasal swabs, lung tissue or cell culture isolates. Currently, the PCR test is rather expensive and therefore it is used more for research than for diagnostic purposes. Serodiagnostic techniques for swine influenza are discussed in Chapter 1.2.2.

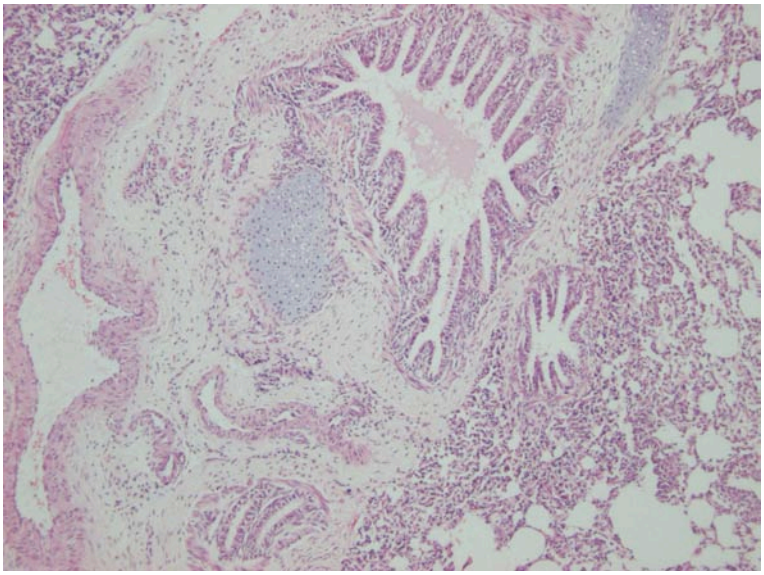

Figure 3. Typical light microscopic lung lesions visible with a hematoxylin and eosin staining (alveolar damage and necrotic debris in bronchi) 24 h after intratracheal inoculation of $10^{6.0}$ EID_{50} swine influenza virus (H3N2).

Prevention and control of swine influenza mainly consists of vaccination. Vaccines contain a representative of a H1N1 and a H3N2 SIV. Vaccines that also contain the more recent H1N2 SIV are not yet licensed in Belgium. The vaccine strains used in

Europe and the United States differ because of different SIV strains circulating in both continents (Olsen *et al.*, 2006). In Belgium the licensed SIV vaccines contain the older human A/New Jersey/8/76 (H1N1) or the porcine Swine/Netherlands/25/80 (H1N1) and A/Port Chalmers/1/73 (H3N2). The A/New Jersey/8/76 H1N1 strain is included in the SIV vaccine from Merial (Gripovac®). The sw/Netherlands/25/80 H1N1 SIV is included in the SIV vaccine from Fort Dodge A.H. (Suvaxyn Flu®). Although the strains used in the SIV vaccines differ antigenically from the currently circulating porcine influenza viruses (de Jong *et al.*, 1999), the vaccines can still offer an outstanding clinical and virological protection. In vaccination-challenge studies, protection of pigs against SIV challenge is correlated with the pre-existing titre of serum hemagglutination inhibition (HI) antibodies and the protection is better after an intranasal than after an intratracheal challenge (Heinen *et al.*, 2001; Van Reeth *et al.*, 2001a; Van Reeth *et al.*, 2001b).

The economical significance of swine influenza and the cost-benefit of vaccination against swine influenza have not yet been thoroughly studied. The retarded weight gain resulting in an increase in the number of days to reach market weight is probably the most important economic repercussion of swine influenza (Heinen *et al.*, 2001).

1.2 THE SEROLOGICAL DIAGNOSIS OF SWINE INFLUENZA

1.2.1 Humoral immune response after swine influenza virus infection

The immunity consists of the innate immunity, which includes amongst other things IFN-α, and the adaptive immunity. The humoral immune response is part of the adaptive immunity against many pathogens including influenza viruses. In contrast to the innate immunity, the adaptive branch of the immune system has a high specificity for the offending pathogen and it has a memory. A principal component of the humoral immune system is the plasma cell, which produces antibodies against pathogens. When bone marrow derived B-cells are activated by antigen recognition and T-helper cells they evolve into antibody secreting plasma cells or form a pool of memory cells. Antibodies are a heterogeneous family of proteins in the body and they account for approximately 20% of the plasma proteins. A typical mammalian antibody molecule consists of 2 identical heavy and 2 identical light chains. There are 5 different classes of immunoglobulins (IgG, IgA, IgM, IgD and IgE) based on differences in the heavy chains. IgG is the predominant antibody class in serum. IgM is a pentamer of 5 antibodies and is the first antibody class that is secreted after an infection. IgA is secreted in mucosal secretions as a dimer of 2 immunoglobulins connected by a J chain. Antibodies contribute to the elimination of a viral infection by 1) binding to, followed by destruction of, infected cells and 2) binding with released progeny virus which inhibits the spread of the infection, termed virus neutralization (Goodman, 1991).

The humoral immune response induced by a SIV infection has been studied extensively. Most antibodies produced during a SIV infection are directed against the HA, NA, NP and M protein. Antibodies to the HA can block attachment of the virus to the host cell receptors and thus neutralize viral infectivity. Antibodies to the NA, NP and M proteins cannot prevent the initiation of infection, but they can mediate destruction of infected cells and antibodies to the NA also interfere with the release of virus particles from an infected cell (Olsen *et al.*, 2006). After a primary SIV infection, antibodies of the IgM isotype are first produced followed by IgG antibodies (Kim *et al.*, 2006). IgM antibodies are undetectable one month after infection whereas IgA and IgG antibodies are still present. In this way detection of IgM antibodies could be used as an alternative method instead of paired sera for the serological diagnosis of a recent SIV infection (Lee *et al.*, 1995; Heinen *et al.*, 2000). Hemagglutination

inhibiting antibodies in serum can be detected starting from 1 week after infection, peak at 2-3 weeks after infection and decline from 8 weeks after infection (Heinen *et al.*, 2000; Van Reeth *et al.*, 2006). Using a sensitive immunoperoxidase monolayer assay (IPMA) test, antibodies were detected in serum as early as 3 days post inoculation (Direksin *et al.*, 2002). In several studies the local antibody response in broncho-alveolar lavage fluid (BALF) and nasal lavage fluid (NLF) of experimentally SIV inoculated pigs was compared with the serum antibody response. IgA was the predominant antibody isotype in both BALF and NLF whereas IgG was the predominant antibody isotype in serum (Heinen *et al.*, 2000; Larsen *et al.*, 2000; Kitikoon *et al.*, 2006). Larsen *et al*. (2000) could demonstrate antibody-secreting cells in nasal mucosal tissue, which proves that antibodies are also locally produced in the respiratory tract of pigs.

1.2.2 Tools for serological diagnosis of swine influenza

Since clinical symptoms and lesions of swine influenza are not pathognomonic, laboratory testing is required to confirm diagnosis. Different diagnostic tools are at our disposal as reviewed by Janke (2000) and discussed in Chapter 1.1.3. Although virus isolation is the most accurate diagnostic technique for detection of an ongoing influenza virus infection in swine, serologic techniques might still be useful for laboratories that do not have the possibilities for cell culture or incubation of eggs, for determination of the immunological status of swine against SIV, to optimize vaccination schedules and for seroprevalence studies. Serological data, however, are difficult to interpret because of antigenic differences between the SIV strain that infected the pig and the SIV strain used in a serological test. For serological diagnosis of SIV, the use of paired acute and convalescent sera, 3 to 4 weeks later, is necessary. Since the acute serum might already contain antibodies from earlier infections or vaccination, only a four-fold or higher rise in antibodies in the convalescent serum is conclusive for the diagnosis. The HI test, virus neutralization (VN) test, IPMA and the enzyme-linked immuno sorbent assay (ELISA) are the most commonly used tests to detect SIV specific antibodies and will be briefly discussed below. The properties of the different serological tests are summarized in Table 2.

Table 2. Strengths and weaknesses of the current serological tests for the detection of antibodies against swine influenza virus.

characteristic	HI[(1)]	VN[(2)]	IPMA[(3)]	ELISA[(4)]
sensitivity	++	+++	++	-
subtype specificity	++	++	-	+/-
convenience	+	-	+	+++
correlation of titre with protection	++	++	-	-
labour intensive	+	++	+	-
suitable as standard test	+++	+	-	-

+++: very high, ++: high, +: moderate, +/-: depends on test kit, -: low
(1) hemagglutination inhibition test, (2) virus neutralization test, (3) immunoperoxidase monolayer assay, (4) enzyme-linked immuno sorbent assay,

The HA of influenza viruses can bind to red blood cells (RBC) of mammalian or avian origin and this property is used in the HI test. Consequently, this test detects antibodies against the HA of the virus. Serum samples have to be pretreated by adsorption to RBC to remove non-specific agglutinins and by receptor destroying enzyme from *Vibrio cholerae* to inactivate non-specific inhibitors of the agglutination. Next, serum samples are serially 2-fold diluted, 4 hemagglutinating units of influenza virus are added, and the mixture is incubated for one hour. A RBC suspension is added and one hour later HI titres can be recorded. The HI titre is the reciprocal of the last sample dilution that completely inhibited hemagglutination (Webster *et al.*, 2005). The HI test is very accurate and is the "gold standard" for serodiagnosis of swine influenza. Critical in this test is the antigenic agreement between the SIV strain used in the test and the SIV strain against which antibodies are detected. This can be a disadvantage when it is important to detect antibodies against any SIV but it is an advantage for seroprevalence studies to know which subtypes and strains are circulating in the population. Separate HI tests against representatives of the predominant subtypes endemic in the region of sampling have to be performed. In the European situation this requires 3 HI tests with representatives of H1N1, H3N2 and H1N2 SIV. The HI test can adequately discriminate between European H1N1, H3N2 and H1N2 SIV since they have antigenically different HAs (Van Reeth *et al.*, 2006). In the United States the situation is different because the American H1N1 and H1N2 SIV share a similar "classical" H1 and the HI test can not discriminate between both (Long *et al.*, 2004).

The VN test is very labour intensive and technically complicated but very suitable for the detection of antibodies against influenza viruses (Benne *et al.*, 1994). Serially two-fold diluted heat inactivated serum samples are made in duplicate and incubated with a standardized amount of virus prior to the addition to Madin-Darby Canine kidney (MDCK) cells. The neutralization can be detected after 3-4 days of incubation by the presence of cytopathogenic effect (CPE) under an inverted light microscope. The VN titre is the reciprocal of the dilution where 50% of the wells shows CPE (Webster *et al.*, 2005). For neutralization of 1 infectious influenza virion about 70 antibody molecules are necessary (Outlaw and Dimmock, 1991). The VN test detects antibodies against the HA since such antibodies prevent infection of the cell and consequently VN titres are correlated with protection against disease. The VN test has advantages over the HI test since it is more sensitive (Gross and Davis, 1979). The VN test is time consuming because CPE can only be read several days after the virus-antibody mixture has been inoculated on the cells. This incubation period can be shortened when infection of the cells is judged by an immunoperoxidase staining after only 1 day of incubation in stead of the appearance of CPE (Okuno *et al.*, 1990). The antibodies used for such staining have to recognize the influenza virus strain used in the test, and to circumvent this problem, monoclonal antibodies against the conserved NP are useful (Kim *et al.*, 2006).

The IPMA, which is rarely used for the serodiagnosis of SIV, is an influenza subtype unrestricted test. In this test, MDCK cells in 96-well plates are infected with influenza virus, incubated for a few days and fixed with ethanol. Plates can be stored at -20°C until use. Next, dilution series of the serum samples are added and following incubation a peroxidase-labelled anti-swine antibody is added. The reaction is visualized by addition of a substrate. The IPMA titre is the reciprocal of the highest dilution with positive cells. The test probably detects antibodies against NP because the IPMA yields light microscopically the same picture of a cytoplasmatic staining as a staining with a monoclonal anti-NP antibody of influenza infected MDCK cells. IPMA titres are not correlated with protection against disease. This test is positive earlier than the HI test, at 3 versus 7 days post inoculation, and IPMA titres are higher than HI titres (Direksin *et al.*, 2002). Another advantage of the IPMA test is that sera do not have to be pretreated as in the HI test. IPMA tests can also be used to detect different isotypes of antibodies by adapting the secondary antibody in the test.

Several commercial ELISA kits are marketed in Europe. Depending on the manufacturer they can be subtype specific and detect antibodies against H1N1 or H3N2 SIV. Often these kits are of American origin and it was not known at the start of this research whether they could also detect antibodies against the H1N1 and H3N2 SIV strains circulating in Europe. An ELISA principle used in other kits is the detection of antibodies against NP. These kits detect antibodies against all SIV subtypes and are species unrestricted (Kim *et al.*, 2006). ELISAs for NP antibodies were originally developed for the detection of antibodies against avian influenza viruses but some are also validated for the detection of antibodies against SIV.

Serological tests do not allow to differentiate between antibodies induced by infection or vaccination although one study shows that this differentiation can be made by an ELISA based on the NS1 protein against which antibodies are present in infected pigs but not in vaccinated pigs, but such test is not yet commercially available (Kim *et al.*, 2006).

1.3 IFN-α AND OTHER CYTOKINES WITH A ROLE IN THE PATHOGENESIS OF SWINE INFLUENZA

1.3.1 Brief introduction on interferon

Interferon (IFN) was first discovered by Isaacs and Lindenmann (1957). They observed that incubation of heat-inactivated influenza virus with chicken chorio-allantoic membranes resulted in the release of a factor that interfered with the growth of live virus. Hence, this factor was named interferon. A correlation was found between the IFN concentration and its ability to suppress the growth of influenza virus (Isaacs *et al.*, 1957). Interferons are a broad family with many members. They are classified as type I or type II IFN (Sen and Lengyel, 1992) or type III IFN. Type I IFN (virus-induced IFN) contains IFN-α, IFN-β, IFN-ω, IFN-κ, IFN-δ, IFN-ε and IFN-τ. IFN-α and IFN-β were originally named leukocyte and fibroblast IFN because the hypothesis was that these cells were the main producers of IFN-α and IFN-β, respectively. The porcine IFN-α multigene family contains 14 functional IFN-α genes and 2 IFN-α pseudogenes (Cheng *et al.*, 2006). In contrast with IFN-α, there is only 1 IFN-β subtype (Artursson *et al.*, 1992). IFN-γ is the only representative of type II IFN (immune IFN). Type III IFN was described more recently and comprises IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) (Kotenko *et al.*, 2003). Type III IFN is also induced upon virus infection or by type I IFN and is regulated by the same mechanism as IFN-α/β (Onoguchi *et al.*, 2007). IFN-α and IFN-β have a strong antiviral activity whereas IFN-λ and IFN-γ have a limited antiviral activity (Ank *et al.*, 2006). Type I IFNs other than IFN-α/β, play ill-defined roles such as establishing uterine receptivity to implantation in mammals (Bazer *et al.*, 2008).

1.3.2 Induction of IFN

Type I IFN is produced by virus infected cells. Virus specific RNA (5' triphosphate moieties of single-stranded RNA and double-stranded RNA) is recognized by the cell as non-self and initiates interferon production (Bowie and Fitzgerald, 2007). The intracellular cascade for IFN induction is complex and induction of IFN-β is best understood. This process involves IFN regulatory factors (IRF) or nuclear factor kappa B (NF-κB). These factors migrate to the nucleus upon stimulation where they bind to a promotor of the IFN-β gene that is activated and

transcripted and subsequently IFN-β protein is synthesized and released from the cell (Haller *et al.*, 2007; Randall and Goodbourn, 2008).

1.3.3 Intracellular signalling and the antiviral effect of IFN-α

Both IFN-α and IFN-β bind to a common heterodimeric type I IFN receptor, consisting of two subunits: IFNAR1 and IFNAR2 (Kim *et al.*, 1997). One subunit of the receptor binds with tyrosine kinase 2 (TYK2) and the other subunit with a janus kinase (JAK1). After binding of IFN on its receptor, these kinases phosphorylate both subunits of the receptor. Next, signal transducer and activator of transcription (STAT) 1 and 2 are phosphorylated and form a dimer that is transported to the nucleus. The dimer associates with IFN regulatory factor-9 (IRF-9) and subsequently the complex binds to IFN-stimulated response elements (ISRE) located in the promotors of IFN-responsive genes. In this way, several hundreds of genes are up-regulated (Randall and Goodbourn, 2008). This pathway is schematically represented in figure 4.

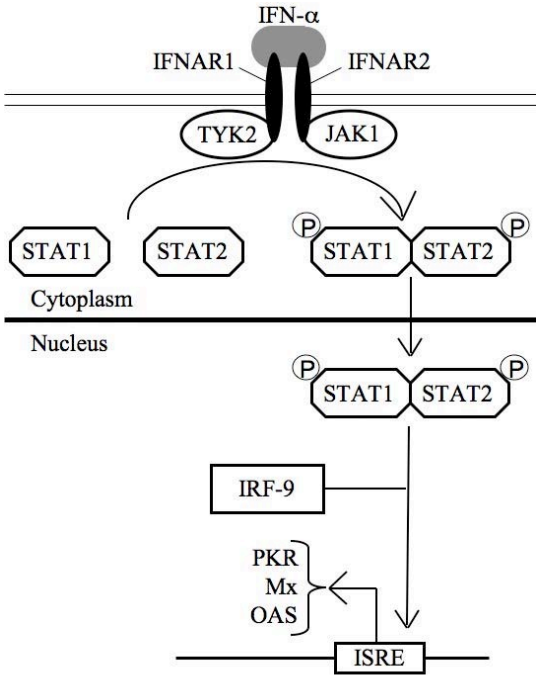

Figure 4. IFN-α signalling cascade (explanation see text). Binding of IFN-α to its receptor activates an intracellular cascade, leading to the production of antiviral proteins (PKR, Mx and OAS).

Some of the proteins induced by this pathway have an antiviral activity. The best characterised are protein kinase R (PKR), 2',5'-oligoadenylate synthetase (OAS) and orthomyxovirus resistance (Mx) protein. Probably there are still other antiviral

pathways since mice with a targeted disruption in these three genes still have a limited antiviral activity against encephalomyocarditis virus (Zhou *et al.*, 1999). PKR is synthesised in cells in an inactive form. After virus infection of the cell, PKR is activated by dsRNA (Clemens, 1997). In its active form it phosphorylates eukaryotic initiation factor 2 (eIF-2), resulting in inhibition of protein synthesis (Rhoads, 1993). Another gene that is up-regulated by the IFN signalling pathway is OAS. OAS activates RNase L that cleaves within ssRNA, such as viral mRNA. Since RNase L has a higher affinity for viral RNA than for cellular RNA (Li *et al.*, 1998), viral protein synthesis is inhibited (Silverman, 2007). The third major antiviral protein is Mx. The exact mechanism of action of Mx is not yet fully understood (Lee and Vidal, 2002; White and Sen, 2009). Mx protein is a GTPase that belongs to the superfamily of dynamin-like GTPases. Probably Mx blocks viral nucleocapsid transport or viral RNA synthesis, depending on whether Mx is present in the cytoplasm or in the nucleus (Samuel, 2001). There are two Mx proteins in mice: Mx1 and Mx2. Mx1 is indispensable for the recovery from influenza in mice and Mx2 is involved in the resistance against vesicular stomatitis virus (Staeheli *et al.*, 1986; Zürcher *et al.*, 1992). In influenza virus-susceptible mice, the Mx1 gene is defective and most inbred strains, such as BALB/c mice, carry non-functional Mx1 alleles, which is probably a founder effect (Staeheli *et al.*, 1988). In humans the homologous genes are MxA and MxB. In pigs, 2 homologous proteins were also identified and named Mx1 and Mx2 (Müller *et al.*, 1992). Cells expressing porcine Mx2 protein had a two-fold reduced titre of progeny virus meaning that porcine Mx2 possesses antiviral activity against influenza (Morozumi *et al.*, 2009).

1.3.4 Systemic effects of cytokines with emphasis on IFN-α

During bacterial infections some components of the bacteria, such as the cell-wall component lipopolysaccharide (LPS) or endotoxin of Gram-negative bacteria exert toxic effects on themselves. Synergistic effects of bacterial LPS and porcine reproductive and respiratory syndrome virus (PRRSV) or porcine respiratory coronavirus (PRCV) in disease induction have also been described (Van Gucht *et al.*, 2003). Therefore, respiratory viruses, together with bacteria, play an important role in multifactorial respiratory disease (Van Gucht *et al.*, 2004). Viruses, like influenza, do not possess such toxins although in earlier days a direct toxic effect of influenza viruses in mice was assumed (Henle and Henle, 1946a; Henle and Henle, 1946b).

Toxic effects of viruses on the body are thus indirect by apoptosis of infected cells (Herold *et al.*, 2008) and the induction of cytokines. The latter was discovered in 1974 when it was found that a flu-like syndrome in different animal species could be induced by injecting dsRNA, an intermediate in the replication of viruses that induces IFN-α (Carter and De Clercq, 1974).

The effect of IFN-α on the body has been investigated by injection of IFN-α preparations in animals, human volunteers or cancer patients. One of the main effects of IFN-α is the induction of fever (Dinarello *et al.*, 1984; Kurokawa *et al.*, 1996). Other effects include chills, myalgia, malaise, headache, fatigue and anorexia (Scott *et al.*, 1981; Gutterman *et al.*, 1982; Segall and Crnic, 1990; Corssmit *et al.*, 1995). A remarkable difference between humans and mice after administration of IFN-α is that humans develop fever while mice do not. This difference is also seen in influenza infections in humans or mice (Valentine *et al.*, 1998). IFN-α also has an effect on the cell profile in the blood: after intramuscular or subcutaneous injection it induces a transient rise in neutrophil counts (Scott *et al.*, 1981; Corssmit *et al.*, 1997).

Other cytokines generate clinical effects comparable to IFN-α. Interleukin-1 (IL-1) for example induces fever, reduced food consumption, weight loss, decreased libido, sleepiness and a decreased locomotor activity (McCarthy *et al.*, 1985; Swiergiel *et al.*, 1997; Larson and Dunn, 2001). Some cognitive functions may also be affected (Larson and Dunn, 2001). Also tumour necrosis factor (TNF) causes anorexia and consequently weight loss. This effect has been well described in mice treated with TNF (Bernstein, 1996). Conversely, another study showed a small effect of a combined treatment with TNF binding protein, anti-IL-6 monoclonal antibodies and IL-1 receptor antagonist resulting in a not significant reduction in food consumption in influenza-inoculated mice (Swiergiel and Dunn, 1999). TNF and IL-6 also play a role in fatigue in cirrhosis patients (Albillos *et al.*, 2006). The general effect of TNF, IL-1 and IL-6 is to induce classic illness behaviour, including somnolence, apathy, fever, and anorexia. Hepatic production of acute phase proteins is a characteristic of many inflammatory conditions and mainly IL-6, TNF and IL-1 induce the production of these proteins (Murch, 1998).

The fever and the behavioural changes caused by cytokines are supposed to be a "highly organized strategy that is at times critical to the survival of the individual if it were living in the wild" (Hart, 1988). The fever increases the efficacy of the host's

immune system and the behavioural changes drive the animal to hide and remain safe while the immune system clears the invading pathogen (Hart, 1988). The mechanisms by which cytokines exert their systemic effects are not completely understood. Cytokines can penetrate the blood-brain barrier by saturable transport systems capable of transferring the cytokines IL-1, IL-6 and TNF from the blood to the brain tissue and consequently these cytokines could affect the brain function (Banks *et al.*, 1995). IFN-α has also been found on sites such as the hypothalamus and the pons, where the blood-brain barrier is more permeable (Dafny and Yang, 2005). A third possibility is that cytokines induce secondary mediators in the organ where they are produced and that these are capable of crossing the blood-brain barrier or alternatively cytokines could stimulate afferent branches of the *Nervus vagus* (Katsuura *et al.*, 1990; Netea *et al.*, 1999; Dantzer, 2001). Cytokines produced locally in infected organs mainly exert effects at the site where they are produced. When massive amounts of cytokines are produced there can be a spill-over into the circulation with possible systemic effects (Openshaw, 2004; Barbé *et al.*, 2009).

1.3.5 Studies on the role of IFN-α in the mouse model of influenza

IFN-α is an important cytokine that is part of the innate immunity against virus infections. IFN-α induces an antiviral state in cells and thus provides an early defence mechanism against many viruses. This property can also be used in bioassays to titrate the amount of bioactive IFN-α present in different biological samples (Meager, 2003).

Traditionally, the laboratory mouse is used as a model to study the pathogenesis of influenza. Our knowledge about the effect of IFN-α on the pathogenesis is to a large extent based on the use of mice or cell cultures. Different tools can be used to this purpose, e.g. a wide variety of neutralizing antibodies, knock-out animals or cell lines. The main disadvantage of these approaches is that mice are not natural hosts for influenza viruses and consequently mouse-adapted influenza viruses, like the A/PuertoRico/8/34 influenza strain, have to be used. Cell lines are easier to manipulate than animals but they are hardly relevant for the *in vivo* situation.

The profile of IFN-α during a murine influenza infection is well known. While the reaction of the body following a bacterial or viral infection is often clinically indistinguishable, only IFN-α is typically associated with viral disease (Gendrel *et al.*, 1999). The interferon inducing capacity of influenza viruses in the lungs of influenza-

inoculated mice is already known for a long time. IFN is produced early after influenza infection. Several studies show maximal lung tissue levels of IFN between 2 and 4 days after intranasal inoculation of influenza and the peak of IFN is accompanied by maximal virus titres (McLaren and Potter, 1973; Wyde *et al.*, 1982). Also tracheo-bronchial washings and BALF of influenza inoculated mice show a similar IFN profile and these samples also contain several other cytokines, such as IL-1α, IL-1β, IL-6, TNF, IFN-γ and granulocyte-monocyte colony stimulating factor (GM-CSF), which show the same profile as IFN (Iwasaki and Nozima, 1977; Vacheron *et al.*, 1990; Hennet *et al.*, 1992; Conn *et al.*, 1995; Peper and Van Campen, 1995). TNF is a key mediator of lung lesions since treatment with TNF antibodies ameliorated gross and histological lung lesions but did not affect influenza lung virus titres (Peper and Van Campen, 1995). The main cytokine producing cells in an influenza infection appear to be monocytes/macrophages as reviewed by Julkunen *et al*. (2000).

Administration of IFN-α neutralizing antibodies is an adequate technique to gain more insight on the role of IFN-α in the pathogenesis of viral infections. This is an approach that was already used about 30 years ago in encepalomyocarditis, herpes simplex, vesicular stomatitis, Newcastle disease or influenza virus inoculated mice (Gresser *et al.*, 1976a; Gresser *et al.*, 1976b; Hoshino *et al.*, 1983). In these studies a partially purified sheep antiserum that was obtained after immunization of sheep with a crude mouse interferon preparation was used. Results of both studies were not univocal. In the study of Gresser *et al*. (1976b) intravenous administration of the interferon antiserum in intranasally influenza inoculated mice did not influence the course of the disease. In the study of Hoshino *et al*. (1983), in contrast, mice were inoculated with influenza virus by aerosol and the interferon antiserum was administered intranasally. In this case the antiserum treated animals survived whereas control animals died and antiserum treated animals had higher lung virus titres. The seemingly contradictory findings of both studies suggest that the inoculation route of the virus and the way in which the interferon antiserum is administered are critical for the outcome of the influenza infection in mice.

More recently, mice deficient in the type I IFN receptor became available. This approach has an advantage over the use of IFN neutralizing antibodies in that the effect of all type I IFNs is blocked. But even in this system the overlap or redundancy

of the effect of different cytokines cannot be prevented. Type I IFN receptor knock-out animals did not show major differences with wild type animals regarding lethality, virus replication, kinetics of the cellular immune response or the ability to maintain an effective recall cytotoxic T-cell response to heterosubtypic challenge (Price *et al.*, 2000). The same receptor deficient mice were also used in a study that focussed on the body temperatures and sleep responses in influenza inoculated animals. Results of that study indicated that type I IFN slightly ameliorates disease symptoms early in infection while exacerbating symptoms later in the infection (Traynor *et al.*, 2007). Inoculation of mice deficient in the STAT1 protein resulted in systemic infections and higher mortality (García-Sastre *et al.*, 1998).

1.3.6 Pigs as a model for human influenza

Bacterial or viral respiratory tract infections occur with a high prevalence in swine, and many commensal bacteria that can induce cytokines harbour in the respiratory tract. Moreover, healthy conventional pigs can have basal levels of cytokines such as TNF or IL-1 in BALF (Van Reeth *et al.*, 1998). Therefore the use of caesarean-derived colostrum-deprived (CDCD) pigs raised under sterile conditions is necessary for the *in vivo* study of the role of cytokines in respiratory infections in swine. CDCD piglets at the age of 3 weeks are fully capable of cytokine production and are also most suitable from a practical viewpoint (Van Reeth *et al.*, 1998).

Intratracheal inoculation of influenza virus seronegative pigs with a high virus dose ($10^{7.5}$ mean egg infectious dose (EID_{50})) results in typical swine flu symptoms, fulminate lung virus titres and high levels of proinflammatory cytokines in BALF, including IFN-α, IL-6, IL-1, and TNF. Afterwards there is a decline and normalisation of these parameters (Van Reeth *et al.*, 1999; Van Reeth *et al.*, 2002b). For a respiratory infection like SIV the best specimen for the determination of cytokines is BALF since this sample contains the highest cytokine levels as compared to serum or the lung tissue (Barbé *et al.*, 2009). Intranasal influenza inoculation leads to a much milder disease with less symptoms, lower lung virus titres and lower cytokine levels. Vaccination against SIV can greatly reduce or prevent virus replication, induction of cytokines and disease (Van Reeth *et al.*, 2002a). This leads to the hypothesis that massive replication of SIV induces high levels of cytokines in the lungs and that these cytokines are responsible for disease symptoms, as is also stated by other authors (Majde, 2000).

Macrophages in an influenza infection are responsible for apoptosis of epithelial cells and are responsible for alveolar damage in murine and human models of adult respiratory distress syndrome (Herold *et al.*, 2008). Both IFN-β and TNF potentiate virus-induced apoptosis, which may prevent infection and replication in surrounding cells because they are dying (Brydon *et al.*, 2005). Macrophages are also essential in controlling swine influenza. Depletion of these cells leads to a lower production of TNF and a higher production of IL-10. Macrophage depleted animals also showed more severe respiratory signs and higher mortality (Kim *et al.*, 2008).

The recent outbreak of the "Mexican flu" originally designated "swine flu" virus in humans underlines the importance of the pig as a possible source of new influenza viruses. Moreover, the same cytokines associated with a SIV infection have been demonstrated in NLF of influenza inoculated human volunteers (Hayden *et al.*, 1998) and humans are susceptible to the same influenza subtypes as pigs. The symptoms and pathology of influenza infections in humans and swine show striking similarities (Kuiken and Taubenberger, 2008). The pig lung also shows more morphological and physiological resemblances, such as lung size and functional capacity, with human lungs than mouse lungs (O'Brien and Zachary, 1996). These pathophysiological resemblances between influenza in pigs and humans point out that the pig is a very useful experimental animal model to study the pathogenesis of influenza viruses. Similarities between pigs and humans in influenza pathology are listed in Table 3.

Table 3. Comparison of human (Hayden *et al.*, 1998; Nicholson, 1998; Kuiken and Taubenberger, 2008) and swine (Van Reeth *et al.*, 1999; Olsen *et al.*, 2006) influenza.

characteristic	swine	humans
influenza subtypes	H1N1, H3N2, H1N2	H1N1, H3N2, H1N2
target organs	upper and lower RT(1)	upper and lower RT
target cells	epithelial cells of RT	epithelial cells of RT
symptoms	abdominal thumping, dyspnoe, fever	sore throat, myalgia, chills, fever
most prominent proinflammatory cytokines	IFN-α, IL-6	IFN-α, IL-6
mortality	<1% at farm level	0,5% – 1%
histopathology	neutrophil infiltration, epithelial cell desquamation	necrosis of alveolar epithelium and desquamation, edema fluid
virus clearance	<1 week	<1 week

(1) respiratory tract

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2.

AIMS OF THE THESIS

Aims of the thesis

Swine influenza is an important respiratory disease of pigs with clinical and immunological resemblances to influenza in humans. Cytokines probably play a role in the development of disease symptoms because strong correlations exist between different cytokines (IFN-α and IL-6) and disease symptoms. Currently, diagnosis of swine influenza necessitates expensive or technically complicated techniques such as virus isolation or hemagglutination inhibition tests. There is a big interest in the use of less complicated tests such as ELISAs for the serological diagnosis of swine influenza.

The first aim of the thesis was to examine whether there are alternatives for the serological diagnosis of swine influenza. Several ELISA kits for the detection of antibodies against swine influenza are commercially available in Europe. They are often based on American virus strains and are not yet validated for the detection of antibodies against the swine influenza virus strains circulating in Europe. The sensitivity and specificity of easily performable commercially available ELISAs were compared with the currently used but labour intensive hemagglutination inhibition test. Therefore, sera of pigs experimentally inoculated with different European swine influenza virus subtypes and animals vaccinated with vaccines against swine influenza were evaluated in both tests.

Secondly, we aimed to describe in more detail the course of different cytokines and a few selected acute phase proteins in swine influenza inoculated animals. Until now, the role of specific cytokines in the pathogenesis could not be investigated because of the lack of cytokine neutralizing antibodies. The second aim of the thesis comprised to investigate specifically the role of IFN-α in the pathogenesis of swine influenza by using swine IFN-α neutralizing monoclonal antibodies in swine influenza virus inoculated gnotobiotic piglets.

3.

THE USE OF COMMERCIAL ELISAS FOR THE SEROLOGICAL DETECTION OF SWINE INFLUENZA

3.1

PERFORMANCE OF A COMMERCIAL SWINE INFLUENZA VIRUS H1N1 AND H3N2 ANTIBODY ENZYME-LINKED IMMUNOSORBENT ASSAY IN PIGS EXPERIMENTALLY INFECTED WITH EUROPEAN INFLUENZA VIRUSES

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Abstract.

The IDEXX Swine Influenza Virus H1N1 and H3N2 ELISAs are used worldwide, but their capacity to detect antibodies to European swine influenza viruses (SIVs) has not been documented. A total of 313 well-defined sera from SIV seronegative pigs and pigs experimentally infected with European SIVs were used to compare the performance of both ELISAs and the hemagglutination inhibition (HI) test. The ELISAs largely failed to detect pigs that had been infected with H1N1 (0/42 positive in H1N1 ELISA) or H3N2 only (9/18 positive in H3N2 ELISA; group 1). Higher ELISA detection rates were found after consecutive infection of pigs with either H1N1 or H3N2 and one other subtype (7/40 and 11/22 positive in H1N1 and H3N2 ELISA, respectively; group 2). Of 39 pigs that had been vaccinated twice with one of 4 commercial SIV vaccines (group 3), 25 tested positive in the H1N1 and 4 in the H3N2 ELISA. Pigs that had received a single vaccination after a prior infection with H1N1 and/or H3N2 (group 4) were more frequently positive than group 1 or 3 pigs (23/24 and 15/24 positive in H1N1 and H3N2 ELISA, respectively). Both the H1N1 and H3N2 ELISA showed a low sensitivity (39% and 35%, respectively) relative to the HI test. Because pigs in the field are frequently infected and/or vaccinated with multiple SIV subtypes and variants, they are more likely to test positive in the ELISAs. However, the interpretation of ELISA results will be difficult and HI remains the method of choice.

Introduction

"Avian-like" H1N1 and "human-like" H3N2 swine influenza viruses (SIVs) have been enzootic in the European swine population since the 1980s, and an H1N2 reassortant became widespread in the late 1990s. The hemagglutinin (HA) of most H1N2 viruses shows little antigenic and genetic relationship with that of the swine H1N1 viruses, and it has likely been derived from human H1N1 viruses circulating in the 1980s. Still, novel H1N2 reassortants with an avian-like HA have also been reported (Pensaert *et al.*, 1981; Marozin *et al.*, 2002). Genetic analyses have further shown that all 3 SIV subtypes share similar "avian-like" internal genes. All 3 SIV subtypes are widespread in swine-dense regions of Europe, and pigs frequently experience consecutive infections with two or even three different SIV subtypes (Van Reeth *et al.*, 2008). To further complicate matters, part of the swine population is vaccinated against SIV. The SIV vaccines currently commercialized in Europe are inactivated, bivalent vaccines. These vaccines contain various H1N1 and H3N2 influenza strains, some of which are antigenically rather different from the current SIV field strains (Van Reeth *et al.*, 2001a). A trivalent vaccine containing H1N1, H3N2 and H1N2 strains has been recently developed but is not yet registered. The vaccines are used mainly in gilts and sows, which may have been infected with one or more SIV subtypes before the first vaccination or at later time points.

Serologic testing is used for the diagnosis of SIV, to monitor a herd's immune status, to optimize vaccination schedules and for seroprevalence studies. The hemagglutination inhibition (HI) test is most frequently used for SIV serology and remains the "gold standard". The test is highly specific and antibody titres correlate with protection. However, it also yields variable results dependent on the test virus strain and is very labour intensive. Commercial ELISAs for the detection of SIV antibodies have recently become available and they can test 46 samples per 96-well microtitre plate in just a few hours. All necessary reagents are provided with the kit and part of the test can be automated. The IDEXX Swine Influenza Virus H1N1 and H3N2 ELISA were developed in North America and appear to be suitable for detection of antibodies in pigs exposed to North American H1N1 and H3N2 strains. The H1N1 ELISA has shown an excellent specificity and sensitivity in studies with sera from pigs vaccinated with SIV vaccines used in the U.S. (Carman *et al.*, 2001; Erickson *et al.*, 2002). On the other hand, the ELISA was less sensitive than the HI

test in experimental infection studies with U.S. H1N1 viruses (Skibbe *et al.*, 2004; Yoon *et al.*, 2004). The H3N2 ELISA was capable of detecting antibodies in pigs vaccinated with an experimental U.S. H3N2 SIV-derived modified live vaccine, but there is little data on its sensitivity and specificity (Richt *et al.*, 2006). Both tests are also used in veterinary diagnostic labs in Europe and for seroprevalence studies worldwide (Jung *et al.*, 2002; Vengust *et al.*, 2006; Yoon *et al.*, 2006; Böttcher *et al.*, 2007; Poljak *et al.*, 2008). The H1N1 and H3N2 SIV field and vaccine strains in Europe, however, are antigenically and genetically very different from their counterparts in the U.S. (Olsen *et al.*, 2006) and there are concerns about the sensitivity of the ELISAs for the detection of antibodies to European SIVs (Lang *et al.*, 2007). Still, there are no extensive validation studies with sera to European SIVs.

This study aimed to assess the capacity of the IDEXX H1N1 and H3N2 ELISAs to identify pigs infected with European H1N1 or H3N2 SIV or pigs vaccinated with European SIV vaccines. Well-defined sera from previous experimental studies were tested in the ELISAs, as well as in HI tests to European H1N1 and H3N2 SIVs. Because various and complex situations may occur in the field the study included sera from pigs that had been exposed to a) a single SIV infection, b) consecutive infections with two SIV subtypes, c) a double vaccination with commercial SIV vaccines and d) infection followed by a single vaccination.

The serum samples used in this study originated from other published and unpublished studies (Van Reeth *et al.*, 2001a; Van Reeth *et al.*, 2001b; Van Reeth *et al.*, 2006). A total of 313 sera from 106 pigs were examined. The pigs belonged to one of 4 experimental groups that are described below. Tables 1&4, 2&5 and 3&6 show the different subgroups, the number of pigs in each subgroup and the times of blood collection for groups 1&2, 3 and 4, respectively.

Materials and methods

Serum samples

Group 1: Single inoculation with H1N1, H3N2 or H1N2 SIV. Three groups of pigs had been inoculated intranasally with $10^{7.0}$ EID_{50} of sw/Belgium/1/98 (H1N1), sw/Flanders/1/98 (H3N2) or sw/Gent/7625/99 (H1N2). The virus isolates are representative of H1N1, H3N2 and H1N2 viruses currently circulating in the European swine population and they are described in detail elsewhere (Van Reeth *et*

al., 2004). After inoculation, infection was confirmed by virus isolation from nasal swabs. Blood was collected 0, 2, 4, 6 and 8 weeks after virus inoculation.

Group 2: Dual, consecutive inoculations with two SIV subtypes. Four groups of pigs had been inoculated consecutively with two SIV subtypes at a 4-week interval. The subtype combinations included H1N1 followed by H3N2 and vice versa, and H1N1 followed by H1N2 and vice versa. The mode and dose of virus inoculation and the virus isolates were identical to those in group 1 pigs. Infection with both subtypes was confirmed by virus isolation from nasal swabs. Blood was collected 0, 2, 4, 6 and 8 weeks after the first SIV inoculation.

Group 3: Vaccination with commercial SIV vaccines. Three groups of 10 pigs each had been vaccinated twice intramuscularly with an interval of 3 weeks with one of 3 commercial SIV vaccines. The following commercial vaccines were used, the influenza virus strains in each vaccine are given between brackets: Gripovac® (A/New Jersey/8/76 (H1N1) and A/Port Chalmers/1/73 (H3N2); Merial, Lyon, France), Suvaxyn Flu® (sw/Netherlands/25/80 (H1N1) and A/Port Chalmers/1/73 (H3N2); Fort Dodge Animal Health, Madison, New Jersey, USA) and Respiporc Flu® (sw/Belgium/230/92 (H1N1) and sw/Belgium/220/92 (H3N2); Impstoffwerk Dessau-Tornau Biologika GmbH, Dessau-Roßlau, Germany). Another group of 9 pigs had been vaccinated with a trivalent SIV vaccine that is not yet registered. This vaccine contains sw/Haselunne/2617/03 (H1N1), sw/Bakum/1769/03 (H3N2) and sw/Bakum/1832/00 (H1N2) and is referred to as “trivalent vaccine” (Impstoffwerk Dessau-Tornau Biologika GmbH, Dessau-Roßlau, Germany). One group of 10 pigs was left unvaccinated. Blood was collected at the time of the first vaccination, and 3 to 4 weeks after the second vaccination.

Group 4: Inoculation with H1N1 and/or H3N2 SIV followed by a single vaccination with commercial SIV vaccine. Three groups of pigs had been inoculated intranasally with H1N1 SIV (sw/Belgium/1/98), with H3N2 SIV (sw/Flanders/1/98) or with H1N1 followed 4 weeks later by H3N2. Four to 5 weeks after the last inoculation, they were given a single intramuscular vaccination with Gripovac®. One control group was left uninoculated and received a single administration of Gripovac® only.

Enzyme-linked immunosorbent assay

The IDEXX Swine Influenza Virus H1N1 and H3N2 ELISAs (IDEXX Laboratories Inc., Westbrook, Maine, USA) were used according to the manufacturer's instructions. The serum samples were diluted 1/40 in sample diluent and 100 µl of positive and negative controls as well as diluted samples were added in duplicate to the wells. Thereafter, plates were incubated for 30 min. After removing the samples, plates were washed thoroughly and 100 µl of an anti-porcine horseradish labelled conjugate was added to all wells. Plates were again incubated for 30 minutes. Next, substrate (tetramethylbenzidine) was added after a washing step and fifteen minutes later the reaction was stopped with a sodium dodecyl sulphate solution. Next, optical densities (OD) were read at 650 nm in an ELISA plate reader. Results were expressed as a sample to positive (S/P) ratio by using the following formula: (mean sample OD – mean negative control OD)/(mean positive control OD – mean negative control OD). Samples with S/P ratios ≥0.4 were considered to be positive for SIV antibodies. For the H3N2 ELISA, S/P ratios <0.4 but ≥0.3 were classified as suspect. All other S/P ratios were classified as negative. All samples and controls were tested in duplicate and the test was performed at room temperature. Arithmetic mean S/P ratios of positive samples were calculated.

Hemagglutination inhibition test

All serum samples were also tested in the HI test using the same H1N1 and H3N2 strains that were used to inoculate the pigs, namely sw/Belgium/1/98 (H1N1) and sw/Flanders/1/98 (H3N2). Serum samples from group 3 animals were also tested for HI antibodies against H1N1 and H3N2 vaccine strains. The HI test was performed according to standard procedures. Briefly, sera were pre-treated with receptor destroying enzyme from *Vibrio cholerae* (Sigma, St. Louis, Missouri, USA) to remove non-specific inhibitors of the agglutination and with a 50% chicken red blood cell (RBC) suspension to remove non-specific agglutinins. Serial two-fold dilutions (starting at 1:10 dilution) of the pre-treated sera were made in phosphate-buffered saline (PBS). After addition of 4 hemagglutinating units of the respective viruses and one hour of incubation, a 0.5% chicken RBC suspension was added. One hour later, HI titres were recorded as the reciprocal of the last sample dilution that completely inhibited hemagglutination. Geometric mean titres of positive samples were calculated.

Statistical analysis

Standard two-sample Mann-Whitney tests were used to compare ELISA S/P ratios between groups. $P < 0.05$ was taken as the level of significance. Cohen's *Kappa* coefficient was used to calculate agreement between HI test and ELISA.

Results

Performance of the H1N1 ELISA

The results of ELISA and HI tests for H1N1 SIV with sera collected after infection, vaccination or combinations of both are shown in Tables 1, 2 and 3, respectively.

Groups 1 and 2: Inoculation with one or two SIV subtypes (Table 1). All sera collected before SIV inoculation and all but one serum collected after inoculation with subtypes other than H1N1 were negative for H1N1 antibodies in the HI test, whereas all sera collected after H1N1 inoculation were positive. Individual HI antibody titres were between 20 and 160 in the pigs inoculated with H1N1 only or with H1N1 followed by H3N2. Higher antibody titres (80–320) were seen after H1N1 inoculation of previously H1N2 inoculated pigs or vice versa.

All sera collected before inoculation or after inoculation with H3N2 or H1N2 were negative in the ELISA. The sera collected after inoculation with H1N1 only (20 sera in group 1 + 22 sera in group 2) were also negative. With one exception, all H1N1-immune pigs remained negative after subsequent inoculation with H1N2 or H3N2. The pigs inoculated with H3N2 followed by H1N1 also tested negative. In the group inoculated consecutively with H1N2 and H1N1, all 4 pigs were positive at 2 weeks post inoculation with H1N1 and 1 pig was still positive at 4 weeks. Individual S/P ratios of positive pigs were between 0.43 and 0.65. All sera that were positive in the ELISA had HI antibody titres ≥160, though 13 of the total 20 sera with HI titres ≥160 were still negative in the ELISA.

Group 3: Vaccination with commercial SIV vaccines (Table 2). The HI test was negative for all sera collected before vaccination or from unvaccinated pigs. All sera collected 3–4 weeks after vaccination were positive in the HI test against the homologous H1N1 virus and against sw/Belgium/1/98 (Table 2). HI antibody titres to the homologous H1N1 vaccine strains were higher than those to sw/Belgium/1/98.

Table 1. Detection of H1N1 antibodies by commercial H1N1 SIV ELISA and HI test after experimental infection of pigs with one (group 1) or with two different SIV subtypes (group 2).

Group	Virus inoculation(s)	n	No. of pigs with H1N1 antibodies in									
			ELISA (mean S/P ratio)[1]					HI (mean antibody titre)[1]				
			0	2	4	6	8w[2]	0	2	4	6	8w
1	H1N1	5	0	0	0	0	0	0	5 (53)	5 (46)	5 (46)	5 (35)
1	H3N2	4	0	0	0	n.a.[3]	n.a.	0	0	0	n.a.	n.a.
1	H1N2	4	0	0	0	0	0	0	1 (20)	0	0	0
2	H1N1-4w-H3N2	6	0	0	0	0	0	0	6 (57)	6 (63)	6 (80)	6 (71)
2	H1N1-4w-H1N2	5	0	0	0	1 (0.47)	1 (0.43)	0	5 (35)	5 (35)	5 (211)	5 (106)
2	H3N2-4w-H1N1	5	0	0	0	0	0	0	0	0	5 (92)	5 (106)
2	H1N2-4w-H1N1	4	0	0	0	4 (0.54)	1 (0.52)	0	0	0	4 (190)	4 (113)

(1) mean S/P ratio (ELISA) or antibody titre (HI) of the positives only, (2) weeks after first SIV inoculation, (3) not available

The ELISA was negative for all pre-vaccination sera and for sera from unvaccinated controls. Post vaccination sera tested positive for all pigs vaccinated with Gripovac® or Suvaxyn Flu®, for 5 out of 10 pigs vaccinated with Respiporc Flu® and for none of the pigs vaccinated with the trivalent vaccine. S/P ratios ranged between 0.49 and 1.18 and were highest in the pigs vaccinated with Gripovac® (P <0.05).

Table 2. Detection of H1N1 antibodies by commercial H1N1 SIV ELISA and HI test after a double vaccination of pigs with commercial SIV vaccines (group 3).

Vaccine	n	No. of pigs with H1N1 antibodies in:					
		ELISA (mean S/P ratio)(1)		HI (mean antibody titre)(1)			
		before vacc.(2)	after vacc.(3)	before vacc.(2)		after vacc.(3)	
				homol(4)	B/1/98(5)	homol(4)	B/1/98(5)
none	10	0	0	n.a.(6)	0	n.a.(6)	0
Gripovac®	10	0	10 (0.98)	0	0	10 (86)	10 (26)
Suvaxyn Flu®	10	0	10 (0.71)	0	0	10 (80)	10 (30)
Respiporc Flu®	10	0	5 (0.59)	0	0	10 (171)	10 (57)
trivalent vaccine	9	0	0	0	0	9 (109)	9 (27)

(1) mean S/P ratio (ELISA) or antibody titre (HI) of the positives only, (2) at the time of first vaccination, (3) 3 to 4 weeks after second vaccination, (4) HI titre against homologous virus, (5) HI titre against swine/Belgium/1/98, (6) not applicable

Group 4: Inoculation with H1N1 and/or H3N2 SIV followed by a single vaccination with commercial SIV vaccine (Table 3). In the HI test, influenza naive control pigs and pigs previously infected with H3N2 tested negative for H1N1 antibodies before vaccination. A single vaccination resulted in an antibody response in all control pigs and 2 of the 4 H3N2-immune pigs, but antibody titres to sw/Belgium/1/98 remained very low (10–20). All pigs that had been previously infected with H1N1 or with H1N1 followed by H3N2 were HI antibody positive before vaccination and showed a dramatic booster of H1N1 HI antibodies after a single vaccination (320–5120).

Pre-vaccination sera of the influenza naive control pigs and of the pigs previously infected with H3N2 were also negative by ELISA. A single administration of the commercial vaccine resulted in an ELISA antibody response in all control pigs and in 3 out of 4 H3N2-immune pigs. The pigs that had been previously infected with H1N1 or with H1N1 followed by H3N2 were ELISA antibody negative before vaccination,

but all of them became positive after a single vaccination. Individual S/P ratios in the latter groups were between 0.84–1.44 and between 0.69–1.31, respectively, and they were higher than those in the control (0.49–0.86) and H3N2-immune groups (0.39–0.67) (P <0.05). The positive ELISA S/P ratios after vaccination of the H1N1 immune pigs corresponded to high HI antibody titres (320–5120). However, pigs in the control and H3N2-immune groups generally had HI antibody titres ≤20 after the vaccination and they were also ELISA positive.

Table 3. Detection of H1N1 antibodies by commercial H1N1 SIV ELISA and HI test after a single vaccination(1) of pigs with infection-immunity to H1N1, H3N2 or both subtypes (group 4).

Virus inoculation(s) prior to vaccination	n	No. of pigs with H1N1 antibodies in:					
		ELISA (mean S/P ratio)(2)			HI (mean antibody titre)(2)		
		0	2	6w(3)	0	2	6w(3)
none	10	0	10 (0.70)	n.a.(4)	0	10 (20)	n.a.
H1N1	4	0	4 (0.98)	4 (1.12)	4 (95)	4 (2560)	4 (1522)
H3N2	4	0	3 (0.61)	n.a.	0	2 (20)	n.a.
H1N1-4w-H3N2	6	0	6 (1.00)	6 (1.00)	6 (40)	6 (1810)	6 (1280)

(1) the commercial vaccine Gripovac® was used for vaccination, (2) mean S/P ratio (ELISA) or antibody titre (HI) of the positives only, (3) weeks in relation to vaccination, (4) not available

Comparison between HI test and ELISA (Table 7). The overall relative sensitivity and specificity of the H1N1 ELISA was 39% and 99%, respectively. Agreement between ELISA and HI was low as illustrated by the Cohen's *Kappa* coefficient of 0.37. In Belgium, the prevalence of SIV H1N1 antibodies in fattening pigs is about 66.7% (Labarque *et al.*, 2004). This gives a positive predictive value (PPV) of 99% and a negative predictive value (NPV) of 45%.

Performance of the H3N2 ELISA

The results of ELISA and HI tests for H3N2 SIV with sera collected after infection, vaccination or combinations of both are shown in Tables 4, 5 and 6, respectively.

Table 4. Detection of H3N2 antibodies by commercial H3N2 SIV ELISA and HI test after experimental infection of pigs with one (group 1) or with two different SIV subtypes (group 2).

Group	Virus inoculation(s)	n	No. of pigs with H3N2 antibodies in:									
			ELISA (mean S/P ratio)[1]					HI (mean antibody titre)[1]				
			0	2	4	6	8w[2]	0	2	4	6	8w
1	H1N1	5	0	0	0	0	0	0	0	0	0	0
1	H3N2	4	4 (0.94)	4 (0.66)	3 (0.54)	n.a.[3]	n.a.	0	4 (226)	4 (135)	n.a.	n.a.
1	H1N2	4	0	0	0	0	0	0	0	0	0	0
2	H1N1-4w-H3N2	6	0	0	0	1 (0.44)	1 (0.44)	0	0	0	6 (57)	6 (36)
2	H1N1-4w-H1N2	5	0	0	0	0	0	0	0	0	0	0
2	H3N2-4w-H1N1	5	0	1 (0.48)	1 (0.43)	5 (0.78)	4 (0.57)	0	5 (106)	5 (92)	5 (80)	5 (80)
2	H1N2-4w-H1N1	4	0	0	0	0	0	0	0	0	3 (13)	3 (10)

(1) mean S/P ratio (ELISA) or antibody titre (HI) of the positives only, (2) weeks after first SIV inoculation, (3) not available

Groups 1 and 2: Inoculation with one or two SIV subtypes (Table 4). All sera collected before inoculation and sera collected after inoculation with subtypes other than H3N2 were negative for H3N2 antibodies in the HI test, except for 3 out of 4 animals inoculated with H1N2 followed by H1N1, which had very low HI antibody titres (10 – 20). All sera collected after H3N2 inoculation were positive. Individual HI antibody titres were between 80 and 320 in the pigs inoculated with H3N2 only and between 20 and 160 in pigs inoculated with H3N2 in combination with H1N1.

Most preinoculation sera and all sera collected after inoculation with H1N1 or H1N2 were negative in the ELISA. Four of the total 33 preinoculation sera, however, were clearly positive in the ELISA (range 0.83–1.13). All 4 sera were from the singly H3N2-inoculated subgroup in group 1 and S/P ratios decreased after the H3N2 inoculation. Only one of the five singly H3N2-inoculated pigs in group 2 was positive in the ELISA, while 5 out of 5 and 4 out of 5 pigs became positive at, respectively, 2 and 4 weeks after the subsequent inoculation with H1N1. Only one of the 6 H1N1-immune pigs became positive after inoculation with H3N2. Individual S/P ratios of the positive pigs in group 2 ranged between 0.43 and 1.02. All these sera had an HI titre ≥40.

Group 3: Vaccination with commercial SIV vaccines (Table 5). The HI test was negative for all sera collected before vaccination or from unvaccinated controls. All sera collected 3–4 weeks after vaccination were positive in the HI test against the homologous H3N2 virus and against sw/Flanders/1/98 (Table 5). All vaccinated pigs had higher HI antibody titres to the homologous H3N2 vaccine strains than to sw/Flanders/1/98. The ELISA was negative on the pre-vaccination sera and on sera from unvaccinated controls. Only 1 or 2 out of 10 pigs vaccinated with Gripovac®, Suvaxyn Flu® or Respiporc Flu®, and none of the pigs vaccinated with the trivalent vaccine, tested positive. Individual S/P ratios were ≤0.60.

Table 5. Detection of H3N2 antibodies by commercial H3N2 SIV ELISA and HI test after a double vaccination of pigs with commercial SIV vaccines (group 3).

Vaccine	n	No. of pigs with H3N2 antibodies in:					
		ELISA (mean S/P ratio)[1]		HI (mean antibody titre)[1]			
		before vacc.[2]	after vacc.[3]	before vacc.[2]		after vacc.[3]	
				homol[4]	Fl/1/98[5]	homol[4]	Fl/1/98[5]
none	10	0	0	n.a.[6]	0	n.a.[6]	0
Gripovac®	10	0	1 (0.57)	0	0	10 (92)	10 (26)
Suvaxyn Flu®	10	0	1 (0.51)	0	0	10 (113)	10 (43)
Respiporc Flu®	10	0	2 (0.53)	0	0	10 (160)	10 (80)
trivalent vaccine	9	0	0	0	0	9 (74)	9 (32)

(1) mean S/P ratio (ELISA) or antibody titre (HI) of the positives only, (2) at the time of first vaccination, (3) 3 to 4 weeks after second vaccination, (4) HI titre against homologous virus, (5) HI titre against swine/Flanders/1/98, (6) not applicable

Group 4: Inoculation with H1N1 and/or H3N2 SIV followed by a single vaccination with commercial SIV vaccine (Table 6). In the HI test, influenza naive control pigs and pigs previously infected with H1N1 only tested negative for H3N2 antibodies before vaccination. Half of these pigs became positive after a single vaccination, but antibody titres to sw/Flanders/1/98 remained low (10–40). All pigs that were previously infected with H3N2 or with H1N1 followed by H3N2 were HI antibody positive before vaccination and showed a dramatic booster of H3N2 HI antibodies after a single vaccination (160–2560).

Pre-vaccination sera of the influenza naive control pigs and of the pigs previously infected with H1N1 or with H1N1 followed by H3N2 were negative by ELISA. A single administration of the commercial vaccine resulted in an ELISA antibody response in none of the control pigs, only 1 of the H1N1-immune pigs, and 5 out of 6 (H1N1 + H3N2)-immune pigs. Three of the 4 pigs that had been previously infected with H3N2 were ELISA positive before the vaccination and all 4 were positive thereafter. All 3 infection-immune groups had similar ELISA S/P ratios after vaccination, and individual values ranged from 0.40 to 0.91. Except for the serum of the H1N1-infection immune pig, all positive sera had HI antibody titres ranging between 80 and 2560.

Table 6. Detection of H3N2 antibodies by commercial H3N2 SIV ELISA and HI test after a single vaccination[1] of pigs with infection-immunity to H1N1, H3N2 or both subtypes (group 4).

Virus inoculation(s) prior to vaccination	n	No. of pigs with H3N2 antibodies in:					
		ELISA (mean S/P ratio)[2]			HI (mean antibody titre)[2]		
		0	2	6w[3]	0	2	6w[3]
none	10	0	0	n.a.[4]	0	5 (16)	n.a.
H1N1	4	0	1 (0.51)	1 (0.57)	0	0	2 (14)
H3N2	4	3 (0.61)	4 (0.68)	n.a.	4 (135)	4 (640)	n.a.
H1N1-4w-H3N2	6	0	5 (0.65)	4 (0.55)	6 (16)	6 (508)	6 (254)

(1) the commercial vaccine Gripovac® was used for vaccination, (2) mean S/P ratio (ELISA) or antibody titre (HI) of the positives only, (3) weeks in relation to vaccination, (4) not available

Comparison between HI test and ELISA (Table 7). The overall relative sensitivity and specificity of the H3N2 ELISA was 35% and 97%, respectively. Agreement between the two tests was low and similar to that of the H1N1 ELISA (Cohen's *Kappa* coefficient of 0.37). In Belgium, the prevalence of SIV H3N2 antibodies in fattening pigs is about 51.2% (Labarque *et al.*, 2004). This gives a PPV of 92% and a NPV of 59%.

Table 7. Comparison between HI test and ELISA.

	Sensitivity	Specificity	PPV[1]	NPV[2]	Agreement with HI[3]
H1N1 ELISA	39%	99%	99%	45%	0.37
H3N2 ELISA	35%	97%	92%	59%	0.37

(1) positive predictive value, (2) negative predictive value, (3) agreement expressed as Cohen's *Kappa* coefficient

Discussion

This is the first extensive validation study of the IDEXX H1N1 and H3N2 SIV antibody ELISA with well-defined sera from pigs that had been infected with European SIVs or vaccinated with European vaccines. These ELISAs are based on North American H1N1 and H3N2 SIVs that are antigenically different from their European counterparts. This raises the question as to whether the ELISAs are appropriate to detect antibodies against European SIVs and a lack of sensitivity had already been reported in a limited European study (Lang *et al.*, 2007). The present

data shows that the ELISAs frequently fail to identify pigs infected with contemporary European SIVs or pigs vaccinated with commercial European vaccines, especially if they were previously SIV naive. An unexpected specificity problem was also encountered with the H3N2 ELISA, in which 4 out of 103 preinfection/prevaccination sera tested positive. These sera were shown to be negative in 3 other serological assays for SIV, including HI and virus neutralization tests and a subtype-unrestricted immunoperoxidase monolayer assay, and the nonspecific reactions cannot be explained. In contrast with the ELISAs, the HI test showed an excellent sensitivity and specificity in the present study. This is in part related to the use of identical H1N1 (sw/Belgium/1/98) and H3N2 (sw/Flanders/1/98) strains in the test as those used for experimental infection of the pigs. However, these strains are still appropriate for the serological diagnosis of the currently circulating SIVs and they resemble H1N1 and H3N2 SIVs in other European countries. In addition, all pigs vaccinated with commercial SIV vaccines showed HI antibodies to both the homologous vaccine viruses and the heterologous strains used in the test.

Pigs that had been consecutively infected with 2 of the 3 European SIV subtypes were more frequently positive in both ELISAs than singly H1N1- or H3N2-infected pigs. Similarly, most H1N1 and/or H3N2-infection immune pigs tested positive in the ELISAs after a single administration of commercial SIV vaccine. The higher ELISA detection rates are likely due to increased antibody levels to the viral HA, as demonstrated by the high HI antibody titres in most pigs, or to other viral proteins. These observations agree with a study in which only hyperimmune sera, with HI titres ≥1280 to the homologous virus, were invariably positive in the ELISAs (Schmoll *et al.*, 2006). However, the IDEXX H1N1 and H3N2 ELISAs could not discriminate between hyperimmune sera to European H1N1, H3N2 or H1N2 strains in that study. Also, a second commercial ELISA kit showed a similar low specificity and sensitivity as the IDEXX ELISAs. It is noteworthy that in the field, pigs are frequently infected and/or vaccinated with multiple influenza virus subtypes and variants. This may explain why several field studies report relatively high seropositivity rates in the IDEXX ELISAs and, in some cases, a reasonable level of agreement between ELISA and HI test results. In one study in the U.S., for example, there was an overall 85.5% agreement between the IDEXX H1N1 ELISA and the HI test (Skibbe *et al.*, 2004). In a field study in Austria, SIV seroprevalence rates for H1N1 and H3N2 did not differ significantly in the IDEXX ELISAs (38% and 15%, respectively) and in the HI test

(42% and 19%, respectively) (Lang *et al.*, 2007). The same investigators, however, had previously described low sensitivity and specificity of the ELISAs based on results with well-defined sera from experimentally infected animals. In addition, more than 1/3 of the field sera yielded discrepant results in the IDEXX ELISA and the second commercial ELISA kit tested.

The poor sensitivity of the ELISAs may be due to antigenic differences between the strains in the test kits and the European H1N1 and H3N2 SIVs, to a low sensitivity of the tests as such, or to a combination of both. Some of the present results suggest that antigenic differences may play a role. Only one of the 4 SIV vaccines used (Gripovac®) contains a classical H1N1 virus like the IDEXX H1N1 ELISA and pigs vaccinated with this vaccine had highest ELISA positivity rates and S/P ratios. On the other hand, detailed information is lacking about the strains used in the ELISAs and their antigenic relatedness to the SIVs used in the present study, as well as data about the protein specificity of the ELISAs. Unlike the HI test, which exclusively detects antibodies against the distal, most variable part of the viral HA (Poumbourios *et al.*, 1993), indirect SIV antibody ELISAs may also detect antibodies against conserved viral proteins like matrix proteins or the stem of the HA. More technical details and a different experimental set-up are thus needed to determine the reason(s) for the low specificity. Interestingly, a low sensitivity of the IDEXX H1N1 ELISA has also been reported in two independent experimental infection studies with North American, classical H1N1 SIVs from 1992 (Skibbe *et al.*, 2004; Yoon *et al.*, 2004). In these studies the ELISA detected antibodies in only 6 of 72 and 35 of 60 post-infection sera examined, while all sera were positive in HI tests. The ELISA clearly failed to identify H1N1-seropositive animals as effectively as the HI test, especially at the early stage of infection. Other investigators have tested pig hyperimmune sera against 10 antigenically different U.S. H1 SIVs isolated between 1930 and 2004 (Vincent *et al.*, 2006). These sera were obtained by a double vaccination of pigs with inactivated virus followed by a live virus challenge. All sera showed high HI antibody titres (≥640) to the homologous virus, but the ELISA was only marginally above the S/P positive cut-off ratio of 0.4 for some modern H1 strains. The authors therefore concluded that the HI assay with a panel of H1 and H3 viruses is a better diagnostic test for swine influenza than the ELISA.

SIV serology is more challenging now than in the past. Over the last 10 years novel SIV subtypes and reassortants have emerged in both Europe and North

America. Besides, the H1N1, H3N2 and H1N2 SIVs on both continents have a different genetic constellation and antigenically distinct HA proteins (Olsen *et al.*, 2006). The value of antibody ELISAs based on whole SIVs is diminished in this complex epidemiological situation. In fact, there are currently only 2 options for the serological diagnosis of SIV. A first option is to perform HI tests using strains that are antigenically similar to the predominant SIVs in a given region. In most regions of Western Europe, at least 3 separate HI tests including SIVs with an avian-like H1, a human-like H1 and an H3 are required. Though serological cross-reactions can not be completely excluded, the HI test can largely discriminate between antibodies to these 3 HAs in unvaccinated pigs (Van Reeth *et al.*, 2006). A second option is to use one subtype-unrestricted universal antibody ELISA based on conserved influenza virus proteins (Kim *et al.*, 2006). The nucleoprotein (NP) appears to be the best candidate for this purpose (Kim *et al.*, 2006) and several NP-based, usually multi-species ELISA kits are on the market. This second approach is less complicated than the HI test, but it does not provide information about the subtype or antigenic characteristics of the infecting viruses. Caution must be exerted, however, because there is no published data about the performance of such commercial influenza antibody ELISAs with sera from infected or vaccinated pigs. In preliminary investigations with one of these kits and sera from the present study, pigs infected with 1 or with 2 different SIV subtypes (groups 1 and 2) were usually positive. Part of the vaccinated pigs (group 3), in contrast, tested negative and further validation studies are certainly needed. Finally, ELISA tests based on the influenza virus non-structural NS1 protein, which is expressed only during viral replication, appear to be suitable to differentiate pigs vaccinated with current killed vaccines from infected animals (Kim *et al.*, 2006). Such tests are not yet commercially available and they could considerably improve the serological diagnosis of SIV.

In conclusion, the HI test remains the method of choice for the serological diagnosis of SIV, to monitor a herd's immune status or to determine the prevalence of specific SIV subtypes in the European swine population. The IDEXX SIV ELISAs will likely detect part of the SIV-infected and/or vaccinated pigs under field conditions, but the interpretation of positive ELISA test results will be difficult.

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4.

ROLE OF IFN-α AND OTHER CYTOKINES IN THE PATHOGENESIS OF SWINE INFLUENZA

4.1

CYTOKINES AND ACUTE PHASE PROTEINS DURING THE ACUTE STAGE OF A SWINE INFLUENZA VIRUS INFECTION

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Abstract

This study investigated the profile of various (pro)inflammatory cytokines and acute phase proteins (APP) during a swine influenza virus (SIV) infection. Three-week-old caesarean-derived colostrum-deprived (CDCD) piglets were inoculated intratracheally with $10^{7.5}$ EID_{50} Swine/Belgium/1/98 (H1N1) SIV and sacrificed between 0 and 120 hours post inoculation (PI). Broncho-alveolar lavage fluid (BALF), lung homogenates and sera were examined for the inflammatory mediators by bioassay or ELISA. IFN-α, IL-6, IL-1 and TNF peaked in BALF 24–30h PI, when virus titres and symptoms were maximal. IFN-γ and IL-12 but not IL-18 in BALF increased simultaneously, and serum cytokine titres were either undetectable or up to 100-fold lower than those in BALF. The APP C-reactive protein (CRP) and haptoglobin peaked 24h later than the cytokines and reached higher levels in serum than in BALF, while LPS-binding protein (LBP) rose in BALF only. Lung virus titres were tightly correlated with BALF IFN-α, IL-6, IL-1, TNF, IFN-γ and IL-12, as well as with serum levels of IL-6 and the interferons. Disease was correlated with the same cytokines in BALF and in serum, as well as with BALF LBP and serum CRP. We conclude that IFN-γ and IL-12 probably also play a role in SIV pathogenesis and that APP present in serum and BALF are cytokine induced. The pig is a valuable model to further examine the therapeutic potential of cytokine antagonists in influenza infection.

Introduction

Swine influenza virus (SIV) is a major cause of acute respiratory disease in swine (Loeffen *et al.*, 1999). Typical outbreaks are characterized by high fever, depression, anorexia and a laboured abdominal breathing (Shope, 1931; Olsen *et al.*, 2006). SIV replicates in epithelial cells of the nasal mucosa, tonsils, trachea and lungs, which appear to be the major target organ. Fully susceptible influenza-naive pigs usually show extensive gross lung lesions, and microscopic examination of the lungs reveals necrosis and desquamation of the bronchiolar epithelial cell layer and infiltration of neutrophils (Olsen *et al.*, 2006). The infection is generally limited to the respiratory tract and attempts to demonstrate viraemia or virus replication outside the respiratory tract have been largely unsuccessful. The disease can be reproduced experimentally by intratracheal (IT) inoculation of influenza-naive pigs with a high virus dose ($10^{7.5}$ EID_{50}). This results in respiratory and general symptoms, high lung titres of virus ($\geq 10^{8.0}$ $TCID_{50}$/g), several (pro)inflammatory cytokines and >50% of neutrophils in broncho-alveolar lavage (BAL) fluid (BALF) cells within 1 day post inoculation (PI). Previous experimental infection studies with representative H1N1, H3N2 and H1N2 SIV strains showed highly significant correlations between clinical signs and levels of interferon-α (IFN-α), interleukin-6 (IL-6) and tumour necrosis factor (TNF) in BALF (Van Reeth *et al.*, 2002). The correlation between disease and IL-8 or IL-1, on the other hand, was much weaker. The typical symptoms and pathology of influenza in pigs resemble those in naive humans and the cytokine profile in BALF of pigs is similar to that in nasal lavage fluids of experimentally infected human volunteers (Hayden *et al.*, 1998). In addition, pigs are naturally susceptible to the same influenza A virus subtypes as humans (H1N1, H3N2, H1N2) and show physiological and anatomical resemblances to humans. Therefore pigs are a valuable experimental model to study the pathogenesis of influenza and the role of cytokines therein (Kuiken and Taubenberger, 2008).

Apart from the cytokines mentioned higher, other cytokines like IL-12, IL-18 and IFN-γ may also play a role in influenza pathogenesis. IL-12 is a potent regulator of cell-mediated immune responses such as proliferation of and IFN-γ production by T- and NK-cells (Gately *et al.*, 1991; Monteiro *et al.*, 1998). IFN-γ has also numerous immunological functions such as enhancement of MHC expression, and limited antiviral activity. IL-18 is often considered as an "IFN-γ inducing factor" (Konishi *et*

al., 1997). Intranasal inoculation of human volunteers with A/Texas/36/91 (H1N1) resulted in an increase in IFN-γ in nasal lavage fluid 2-5 days PI (Fritz *et al.*, 1999). In mice inoculated with A/PuertoRico/8/34 (H1N1), both IFN-γ and IL-12 were found in BALF 3-7 days PI (Monteiro *et al.*, 1998). Intraperitoneal treatment of mice with anti-IL-12 antibodies resulted in lower IFN-γ titres but higher lung virus titres (Monteiro *et al.*, 1998). A study involving IL-18 knock out mice also pointed towards a role for IL-18 in influenza virus clearance from the lungs and in the induction of IFN-γ (Denton *et al.*, 2007). In the swine influenza model, IL-12 and IL-18 have never been studied and data about IFN-γ are scarce (Jung *et al.*, 2004).

Acute phase proteins (APP) can also play a role in the pathogenesis of respiratory infections. APP such as haptoglobin (HG), lipopolysaccharide (LPS) binding protein (LBP) and C-reactive protein (CRP) are produced by the liver in response to cytokines (Moshage *et al.*, 1988). HG binds free haemoglobin thus removing it from the circulation. LBP binds LPS leading to its neutralization and clearance. CRP has several functions such as macrophage activation and opsonisation. IL-6, IL-1 and TNF appear to be the main APP inducers (Petersen *et al.*, 2004). A number of studies have investigated APP during influenza infections. During experimental influenza virus infection of human volunteers CRP in sera peaked at 3 days PI (Whicher *et al.*, 1985). In people suffering from a natural influenza infection CRP was significantly increased in acute sera compared to convalescent sera taken 4-6 weeks later (Falsey *et al.*, 2001). Another study showed a similar profile with peak serum CRP levels at 3 and 4 days PI followed by a decline and very low levels at 2 weeks PI (Melbye *et al.*, 2004). Increased HG concentrations were found after experimental influenza virus infection of horses (Kent and Goodall, 1991), peaking 7-10 days PI. In horses naturally infected with equine influenza virus, serum amyloid A was increased in the acute stage of the disease (Hulten *et al.*, 1999). To our knowledge APP have not yet been studied in influenza-inoculated pigs.

A more complete picture of the cytokine and APP profile during SIV infection will increase our understanding of the pathogenesis of influenza in both pigs and humans. The first aim of this study was to investigate the profile of IFN-γ, IL-12 and IL-18 as well as of APP (HG, LBP and CRP) during a SIV infection. The second aim was to compare levels of these cytokines and APP in the lungs and in the circulation. Finally, we have investigated correlations between different cytokines, APP, lung virus titres and neutrophil infiltration.

Materials and Methods

Virus

The Swine/Belgium/1/98 (H1N1) SIV strain had been isolated from the lungs of fattening pigs during an acute respiratory disease outbreak. The stock used for inoculation represented the third passage in eggs. Virus inoculations of pigs were performed IT, using $10^{7.5}$ EID_{50} in 3 mL phosphate-buffered saline (PBS), by inserting a 20-gauge needle attached to a syringe in the trachea through the skin cranial to the sternum.

Pigs, experimental design, sampling and BALF cell analysis

Sixteen 3-week-old caesarean-derived colostrum-deprived (CDCD) pigs were used. The pigs originated from 2 sows and were housed in Horsefall-type isolation units with positive-pressure ventilation and fed with ultrahigh-temperature-treated cow's milk supplemented with antibiotics. Two pigs were mock-inoculated with PBS and euthanized 1 day later. The 14 remaining pigs were inoculated IT with SIV and euthanized at 24 h (n=3), 30 h (n=2), 48 h (n=3), 72 h (n=3) or 120 h (n=3) PI. Euthanasia was performed intravenously by injecting an overdose sodium pentobarbital (Natrium Pentobarbital 20%, Kela). A clinical score to assess disease was attributed to each pig just before euthanasia whereby one point was assigned for the presence of each of following signs: anorexia, depression and coughing. A respiration frequency from 60 - 90 respirations per minute added 1 point, and > 90 respirations per minute added 2 points to the clinical score. The Local Ethical Committee of the Faculty of Veterinary Medicine, Ghent University approved this experiment (authorisation reference number EC 2005/88).

Blood samples for serum collection were taken from all pigs at euthanasia. The whole lung was excised and the right lung half was lavaged with cold PBS to obtain BALF as described elsewhere (Van Reeth *et al.*, 1999). Samples from the left lung lobes were pooled and 20% tissue homogenates were prepared for virus titration and quantification of cytokines. BALF was separated into cells and cell-free fluids by centrifugation. Cell-free BALF was concentrated 20 times by dialysis against polyethylene glycol, cleared of residual virus by centrifugation and used to determine cytokines and APP as described further.

BALF cell analysis

Total numbers of BALF cells were counted using a Türk chamber. Cytocentrifuge preparations were stained with Diff-Quik® (Medion Diagnostics) to determine neutrophil numbers.

Virological examination

Virus titrations of lung homogenates were performed in Madin-Darby Canine Kidney cells as described previously (Van Reeth *et al.*, 2002). Virus titres were calculated using the method of Reed and Muench (Reed and Muench, 1938).

Quantification of cytokines

Sera, BALF and lung tissue homogenates were used for quantification of various cytokines. IFN-α, TNF, IL-1 and IL-6 were assayed using bioassays as described earlier (Van Reeth *et al.*, 1999; Van Reeth *et al.*, 2002). In summary, IFN-α was quantified in a cytopathic effect reduction test with Madin-Darby Bovine Kidney cells and vesicular stomatitis virus. TNF titres were determined in a cytotoxicity test in Porcine Kidney subclone 15 (PK(15)) cells and IL-1 activity was measured in a proliferation assay in D10(N4)M cells. IL-6 was assayed in a proliferation assay using B9 cells. All bioassays were run two or three times and geometric mean titres were calculated. Specificity was demonstrated by neutralizing the samples with specific antibodies for IFN-α, TNF and IL-6 or pre-incubation of the D10(N4)M cells with IL-1 receptor antagonist. IFN-γ, IL-18 and IL-12 were determined by ELISAs (Swine IFN-γ ELISA (Biosource), Pig IL-18 ELISA (BenderMed Systems), Porcine IL-12/IL-23 p40 ELISA (R&D Systems)). Detection limits of these tests were 2, 23 and 18 pg/mL, respectively. All samples were tested in duplicate and ELISAs were performed as instructed by the manufacturers.

Quantification of APP

CRP, HG and LBP were measured in serum and BALF. CRP and LBP were quantified by ELISAs (Phase Range Porcine C-reactive Protein Assay, Tridelta Development Ltd. and LBP ELISA, Hycult Biotechnology). HG was measured using a colorimetric assay (Phase Range Porcine Haptoglobin Assay, Tridelta Development Ltd.). Detection limits of CRP, LBP and HG were 47 ng/mL, 1,6 ng/mL and 50

μg/mL, respectively. All samples were tested in duplicate according to manufacturers' instructions.

Statistical evaluation

Spearman rank correlation coefficients (ρ) were calculated to compare individual cytokine and APP levels in the lungs or circulation, lung virus titres, neutrophil numbers in BALF and clinical scores. $P < 0.01$ was taken as the level of significance.

Results

Clinical symptoms, virus titres and lung inflammatory changes

The PBS-inoculated control pigs did not show symptoms, were free of virus in their lungs and had negligible numbers of neutrophils (1×10^6) in BALF. In the SIV inoculated pigs, clinical signs consisted of abdominal thumping, tachypnoea and depression. These symptoms peaked at 24 and 30 h PI and had completely resolved by 72 h PI (Table 1). Lung virus titres were maximal at 24 and 30 h PI ($10^{9.6}$ $TCID_{50}$/g) and showed a substantial decrease at 4 and 5 days PI ($10^{5.8}$ $TCID_{50}$/g). BALF cell numbers were strongly increased at 24 and 30 h PI (peak of 828×10^6). Increased BALF cell numbers were largely due to neutrophil infiltration since the number of monomorphonuclear cells remained rather constant ($\pm 120 \times 10^6$) during the experiment (Table 1). Neutrophil numbers had dropped substantially by 3 and 5 days PI ($8\text{-}20 \times 10^6$), approaching baseline levels.

Profile of cytokines in BALF, serum and lung tissue suspensions

Titres of bioactive IFN-α, IL-6, TNF and IL-1 in BALF, lung tissue suspensions and serum are shown in Figures 1A, 1B and 1C. The profile of IFN-α, TNF, IL-1 and IL-6 in BALF was in agreement with that in previous studies (Van Reeth *et al.*, 1999; Van Reeth *et al.*, 2002). All 4 cytokines were undetectable in all 3 compartments (BALF, serum and lung tissue suspensions) in PBS-inoculated pigs and were strongly increased in BALF 24 and 30 h PI with SIV. At later time points they were at least 20-fold lower or near the detection limit. Titres of IFN-α and IL-6 were about 100 times higher than TNF and IL-1 titres (see insert Fig. 1A). In lung tissue homogenates (Fig. 1B) and serum (Fig. 1C) the cytokine profiles were similar but cytokine titres

were, respectively, about 10 and 100 times lower than in BALF. Only IFN-α and IL-6 were substantially increased in lung tissue homogenates and serum.

Table 1. Evolution of mean clinical score at euthanasia, lung virus titres and inflammatory changes in the lungs after intratracheal inoculation of pigs with swine influenza virus.

h PI	n	Clinical score ± SD	Virus titre ± SD (log10 $TCID_{50}$/g)	Cell numbers in BALF (1) ($x10^6$) ± SD		
				Total	Neutrophil	Mononuclear
0	2	0.0 ± 0.0	<1.7 ± 0	131 ± 18	1 ± 0	130 ± 18
24	3	1.7 ± 1.5	9.6 ± 0.3	458 ± 310	332 ± 336	126 ± 50
30	2	3.5 ± 0.7	9.2 ± 0.2	614 ± 303	481 ± 211	133 ± 92
48	3	1.7 ± 1.2	8.7 ± 0.3	331 ± 131	114 ± 113	217 ± 122
72	3	0.0 ± 0.0	7.5 ± 0.7	118 ± 24	11 ± 3	107 ± 26
120	3	0.0 ± 0.0	5.8 ± 0.6	228 ± 18	15 ± 5	213 ± 20

(1) broncho-alveolar lavage fluid

Titres of IL-12, IFN-γ and IL-18 in BALF, lung tissue suspensions and serum are shown in Figures 2A, 2B and 2C. In the mock-inoculated pigs, only IL-18 was detectable, in all 3 compartments. In BALF, IL-12 and IFN-γ peaked at 24 and 30 h PI and gradually declined thereafter. IL-18 levels, in contrast, were lower at 24 and 30 h PI than at 0 h PI and they increased thereafter. Lung tissue suspensions revealed a similar cytokine profile as BALF, but IFN-γ and IL-18 levels were, respectively, about 2 and 10 times higher. In serum, IL-12 was absent at all time points, and IFN-γ and IL-18 concentrations were, respectively, about 10 and 2 times lower than in BALF. IL-18 dropped below the detection limit in serum at 30 h PI, along with the decrease of IL-18 in the other two compartments.

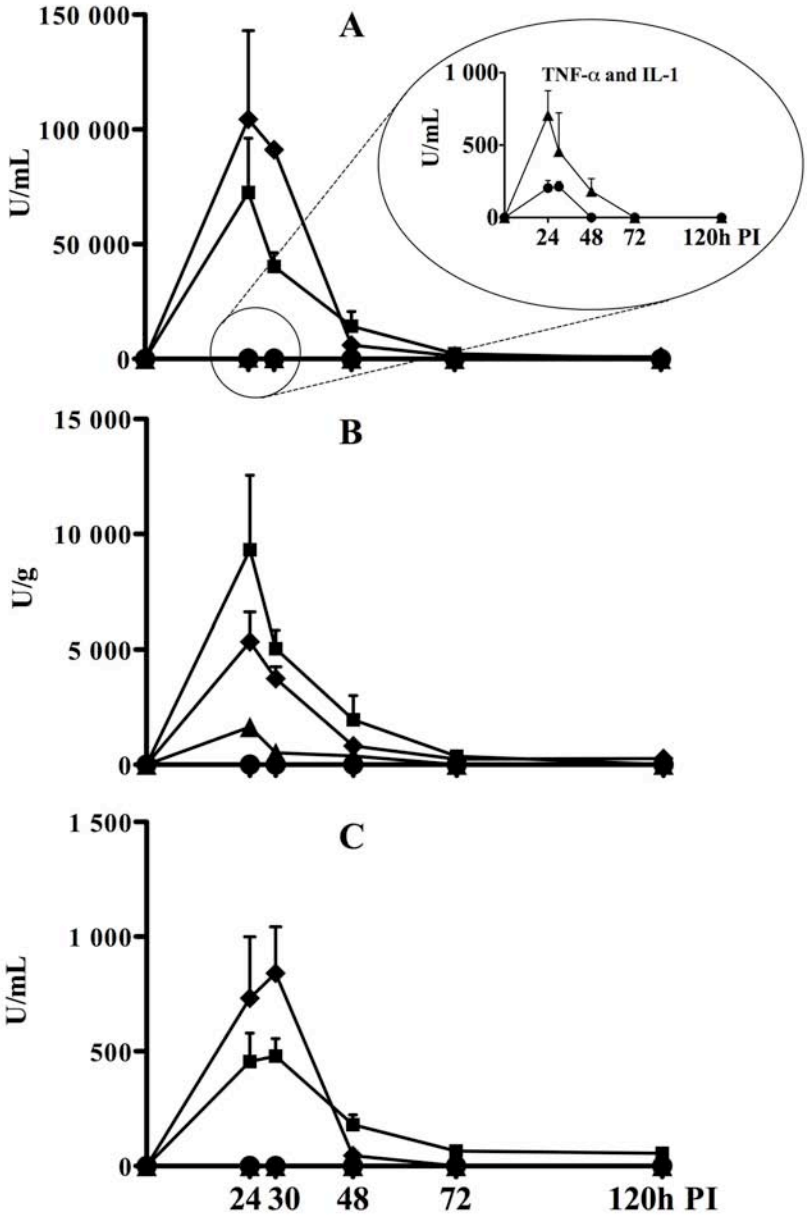

Figure 1. Evolution of IFN-α (■), IL-6 (◆), TNF (▲) and IL-1 (●) in BALF (A), lung tissue homogenates (B) and serum (C) during a swine influenza virus infection. Values are means of 2 or 3 pigs (+ standard error of mean; SEM), and are expressed as biological units/mL (BALF and serum) or per gram (lung tissue homogenates).

Profile of APP in BALF and serum

Levels of LBP, HG and CRP were only examined in BALF and serum (Fig. 3A and 3B). The PBS-inoculated control pigs had low HG and LBP levels in BALF and undetectable CRP. Sera of PBS-inoculated control pigs showed undetectable or low levels of CRP and HG, but substantial levels of LBP. In SIV-inoculated pigs, concentrations of APP in BALF peaked at 30 h PI (LBP) or 48 h PI (HG and CRP)

and decreased thereafter. In serum, CRP and HG showed a peak at 48 h PI, while LBP did not change much. APP levels in serum were roughly 4 times higher than in BALF.

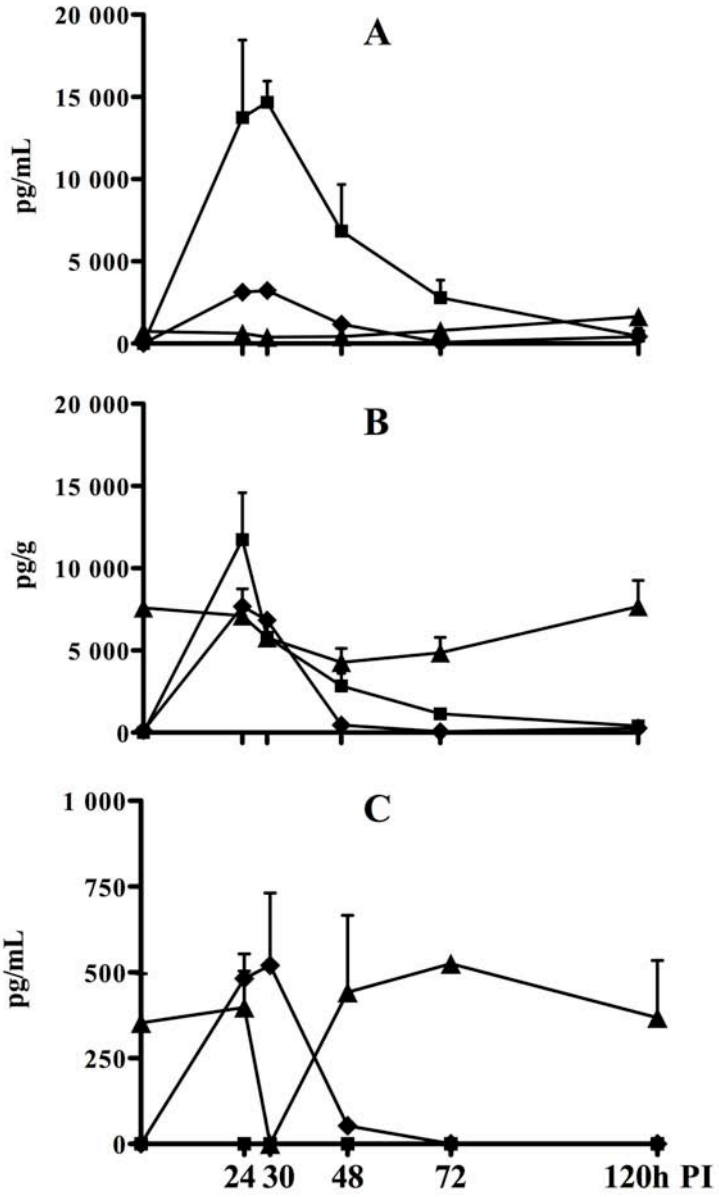

Figure 2. Evolution of IL-12 (■), IFN-γ (◆) and IL-18 (▲) in BALF (A), lung tissue homogenates (B) and serum (C) during a swine influenza virus infection. Values are means of 2 or 3 pigs (+ SEM), and are expressed as pg/mL (BALF and serum) or pg/g (lung tissue homogenates).

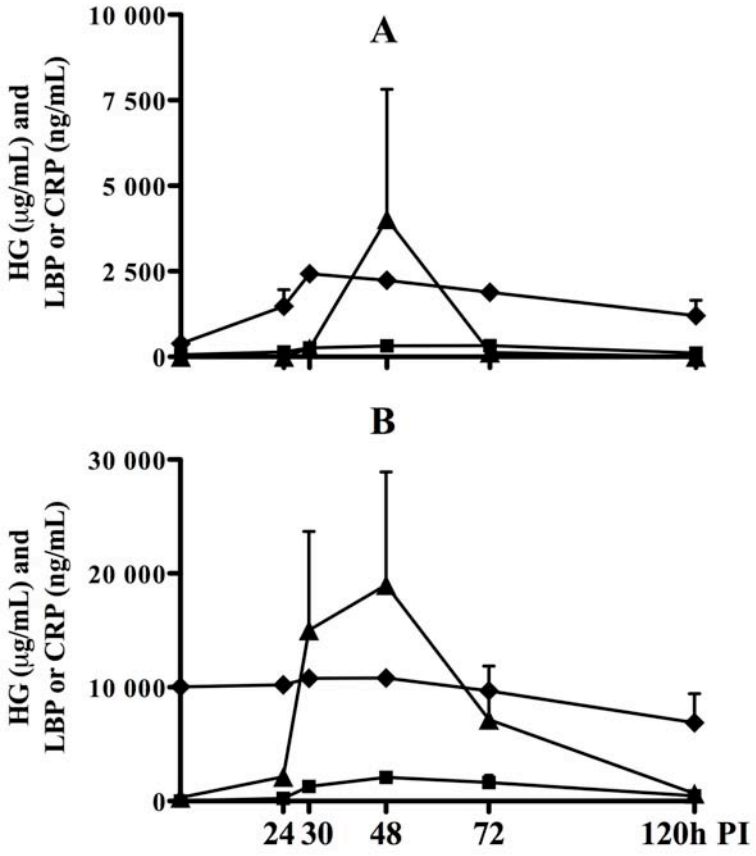

Figure 3. Evolution of HG (■), LBP (◆) and CRP (▲) in BALF (A) and serum (B) during a swine influenza virus infection. Values are means of 2 or 3 pigs (+ SEM), and are expressed in µg/mL (HG) or ng/mL (LBP and CRP).

Correlations between parameters

Correlations between lung virus titres, neutrophil infiltration in BALF and clinical scores on the one hand and cytokines and APP in BALF or serum on the other hand are described in Table 2 and 3, respectively. Virus titres and neutrophil percentages in BALF were significantly correlated with each other and also with all cytokines in BALF, except for IL-18. Disease, assessed by clinical scores, correlated significantly with lung virus titres, neutrophil infiltration and all cytokines in BALF although there was a negative correlation with IL-18. Of the APP in BALF only LBP correlated with disease. The strongest correlations were found between virus titres and IFN-α (ρ = 0.962) or IL-6 (ρ = 0.940). Cytokines in BALF (except IL-18) were also significantly correlated with each other with a Spearman rank correlation coefficient (ρ) of at least

Table 2. Correlation [(1)] between cytokines and acute phase proteins (APP) in broncho-alveolar lavage fluid (BALF), lung virus titres, neutrophil infiltration in the lungs and disease.

	IFN-α	IL-6	IL-1	TNF	IFN-γ	IL-18	IL-12	HG	CRP	LBP	virus	neutro	disease
IFN-α	1	0.920 [(2)]	0.812 [(2)]	0.899 [(2)]	0.856 [(2)]	-0.530	0.925 [(2)]	0.386	0.275	0.608	0.962 [(2)]	0.861 [(2)]	0.738 [(2)]
IL-6	-	1	0.777 [(2)]	0.918 [(2)]	0.932 [(2)]	-0.533	0.893 [(2)]	0.274	0.178	0.670 [(2)]	0.940 [(2)]	0.855 [(2)]	0.819 [(2)]
IL-1	-	-	1	0.787 [(2)]	0.766 [(2)]	-0.390	0.717 [(2)]	-0.069	-0.016	0.243	0.788 [(2)]	0.733 [(2)]	0.689 [(2)]
TNF	-	-	-	1	0.901 [(2)]	-0.573	0.870 [(2)]	0.193	0.164	0.534	0.912 [(2)]	0.801 [(2)]	0.815 [(2)]
IFN-γ	-	-	-	-	1	-0.486	0.814 [(2)]	0.119	0.137	0.561	0.884 [(2)]	0.799 [(2)]	0.717 [(2)]
IL-18	-	-	-	-	-	1	-0.603	-0.332	-0.591	-0.506	-0.549	-0.541	-0.565 [(2)]
IL-12	-	-	-	-	-	-	1	0.355	0.430	0.766 [(2)]	0.889 [(2)]	0.920 [(2)]	0.843 [(2)]
HG	-	-	-	-	-	-	-	1	0.558	0.437	0.314	0.218	0.571
CRP	-	-	-	-	-	-	-	-	1	0.481	0.160	0.354	0.463
LBP	-	-	-	-	-	-	-	-	-	1	0.600	0.759 [(2)]	0.689 [(2)]
virus	-	-	-	-	-	-	-	-	-	-	1	0.860 [(2)]	0.691 [(2)]
neutro	-	-	-	-	-	-	-	-	-	-	-	1	0.704 [(2)]
disease	-	-	-	-	-	-	-	-	-	-	-	-	1

(1) Spearman's ρ correlation coefficient was used, (2) Correlation is significant at the 0.01 level

Table 3. Correlation [1] between cytokines and acute phase proteins (APP) in serum, lung virus titres, neutrophil infiltration in the lungs and disease.

	IFN-α	IL-6	IL-1	TNF	IFN-γ	IL-18	IL-12	HG	CRP	LBP	virus	neutro	disease
IFN-α	1	0.821 [2]	n.a. [3]	n.a.	0.894 [2]	-0.287	n.a.	0.444	0.524	0.258	0.892 [2]	0.823 [2]	0.744 [2]
IL-6	-	1	n.a.	n.a.	0.914 [2]	-0.504	n.a.	0.308	0.380	0.198	0.871 [2]	0.895 [2]	0.811 [2]
IL-1	-	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
TNF	-	-	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
IFN-γ	-	-	-	-	1	-0.408	n.a.	0.258	0.424	0.280	0.906 [2]	0.770 [2]	0.767 [2]
IL-18	-	-	-	-	-	1	n.a.	-0.221	0.000	-0.022	-0.188	-0.388	-0.456
IL-12	-	-	-	-	-	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
HG	-	-	-	-	-	-	-	1	0.637 [2]	-0.203	0.339	0.412	0.429
CRP	-	-	-	-	-	-	-	-	1	0.136	0.476	0.357	0.629 [2]
LBP	-	-	-	-	-	-	-	-	-	1	0.270	0.271	0.251
virus	-	-	-	-	-	-	-	-	-	-	1	0.860 [2]	0.691 [2]
neutro	-	-	-	-	-	-	-	-	-	-	-	1	0.704 [2]
disease	-	-	-	-	-	-	-	-	-	-	-	-	1

(1) Spearman's ρ correlation coefficient was used, (2) Correlation is significant at the 0.01 level, (3) not applicable

0.717. IL-18 was negatively but not significantly correlated with all parameters examined. Of the APP, only LBP correlated significantly with other parameters, namely IL-6, IL-12 and neutrophil infiltration.

In serum, IL-1, TNF and IL-12 were below the detection limit and correlation coefficients for these cytokines could not be calculated. As for BALF, there were strong correlations between lung virus titres, neutrophil infiltration or disease on the one hand and IFN-α, IFN-γ and IL-6 on the other hand and between those cytokines ($\rho \geq 0.770$). IL-18 was negatively but not significantly correlated with other parameters. The only significant correlation for APP in serum was between HG and CRP and between CRP and disease.

Correlations between cytokines in BALF and APP in serum were also calculated (data not shown). The only significant, but negative, correlation existed between IL-18 in BALF and CRP in serum ($\rho = -0.681$).

Discussion

The present study confirms and adds to our previous studies on the cytokine profile in BALF during experimental SIV infection. Those previous studies showed significant increases in IFN-α, TNF, IL-6 and IL-1, and the first three cytokines in particular were strongly correlated with virus replication and disease (Van Reeth *et al.*, 2002). The present study demonstrates a similar secretion profile and correlations for IL-12 and IFN-γ, but not for IL-18. We also found increases in the APP HG, LBP and CRP but, unlike for the cytokines, their levels were higher in serum than in BALF and they peaked 24 h later than the cytokines. In addition, APP were not directly correlated with virus replication and correlations with disease were rarely significant.

IFN-γ was detected in both BALF and serum 24 h PI with SIV. This finding contrasts with two other studies on IFN-γ during experimental SIV infection. Infection with a Korean H1N1 SIV resulted in a small and non-significant increase in ELISA IFN-γ titres in BALF, at 7 days PI only (Jung *et al.*, 2004). Similarly, serum ELISA titres of IFN-γ did not change after infection with a US H3N2 SIV isolate (Wesley *et al.*, 2006). Several factors may account for the higher and more rapid IFN-γ response in our study. We used a higher virus inoculation dose than in both other studies, and intratracheal instead of intranasal inoculation, which results in a

higher virus load in the lungs. Unlike Jung *et al.* (2004), we used concentrated BALF, which increases the sensitivity of cytokine detection, and we focused on earlier times PI than in both other studies. Furthermore, the profiles of IFN-γ and IL-12 in BALF in our pig model of influenza largely agreed with those in BALF of BALB/c mice and nasal lavage fluid of human volunteers, respectively (Monteiro *et al.*, 1998; Fritz *et al.*, 1999).

IL-18 was unexpectedly present in BALF, lung homogenates and serum of PBS-inoculated controls and decreased after SIV inoculation. Both findings are difficult to explain. It is known that IL-18 is expressed intracellularly as a precursor form and that IL-1β converting enzyme (ICE) cleaves the intracellular pro-IL-18 protein into mature IL-18 which is then released from the cell (Ghayur *et al.*, 1997). The commercial ELISA used in our study will most likely detect the extracellular, mature form of IL-18, though the manufacturer could not provide any information on this issue. IL-18 seems to be constitutively expressed in rats and humans. In one study in rats, high ELISA levels of IL-18 were found in lung homogenates (Jordan *et al.*, 2001) and pro- and mature IL-18 was detected in freshly isolated human peripheral blood mononuclear cells (PBMC) (Puren *et al.*, 1999). IL-18 levels around 700 pg/mL were also found in BALF of uninfected one-day-old gnotobiotic pigs using a non-commercial ELISA. BALF IL-18 levels in these piglets, however, were significantly increased at 2 and 4 weeks PI with *Mycoplasma hyopneumoniae* (Muneta *et al.*, 2008). A transient increase in IL-18 ELISA concentrations was also observed in BALF of other animal species following experimental infection with various pathogens. This was the case, for example, at 3 days PI of mice with herpes simplex virus type 1 (HSV-1), while IL-18 was undetectable at 1 or 7 days PI or in uninoculated controls (Reading *et al.*, 2007). In in vitro studies human PBMC-derived macrophages were shown to secrete IL-18 after inoculation with H3N2 influenza virus (Pirhonen *et al.*, 1999). There are, however, no in vivo studies of the kinetics of IL-18 during influenza virus infection of humans or experimental animals and the reason for the decreased IL-18 levels in our SIV inoculated pigs remains uncertain. The proteins of certain viruses like poxviruses or papillomaviruses are known to counteract IL-18 by their capacity to bind to it (Xiang and Moss, 1999; Cho *et al.*, 2001). Another possible way to decrease IL-18 levels is the induction of IL-18 binding protein (BP) in response to IFN-α, which occurs in chronic hepatitis C

patients (Kaser *et al.*, 2002). PBMC-derived human macrophages treated with IFN-α were also shown to synthesize less IL-18 protein. Finally, it cannot be excluded that concentrations of mature IL-18 are affected by changes in the concentration of ICE, which is also involved in the secretion of IL-1β and in apoptosis (Lynch *et al.*, 1997). Whatever the reason for the decreased IL-18 levels in our study, our data do not fit with a role for IL-18 in the clearance of influenza virus or induction of IFN-γ as has been described in the mouse model of influenza (Denton *et al.*, 2007).

Most cytokines produced during a SIV infection were found at higher levels in BALF than in lung homogenates, and only TNF and IFN-γ were detected at similar levels in both types of sample. Most important, only the interferons and IL-6 reached detectable levels in serum and these were 5 to 100-fold lower than in BALF. These findings agree with observations after experimental H1N1 influenza virus infection of human volunteers (Hayden *et al.*, 1998). The cytokine responses in plasma or serum of these humans were either 100-fold lower than those in nasal lavage fluids, which was the case for IL-6 and TNF, or undetectable. Increased plasma levels of various cytokines (IL-6, IL-8, IFN-inducible protein 10 (IP-10) and monokine induced by IFN-γ (MIG)) have also been reported in very severe cases of human influenza virus infection, for example in patients that were hospitalized with human H1N1 influenza virus infection (Lee *et al.*, 2007) and in those with highly pathogenic avian H5N1 influenza virus (Peiris *et al.*, 2004; Gambotto *et al.*, 2008). Local cytokine concentrations for those patients were not examined but their plasma levels were tightly correlated with viral RNA levels in nasopharyngeal or throat swabs, as well as with clinical severity. Though the H5N1 virus may also replicate in non-respiratory organs, the lungs or other sites of the respiratory tract are the major site of replication and pathology for influenza viruses in mammals. All evidence points towards an initial production of cytokines in the lungs and it is accepted that cytokine levels in the circulation represent only the spill-over from the respiratory tract. It is logical, therefore, that considerable plasma or serum levels are only detectable in “high risk” patients with massive virus replication in the respiratory tract and prolonged severe illness. This is fully supported by our comparative investigations of BALF, lung tissue and serum in pigs. As stated by Openshaw in a paper about cytokine measurement in blood samples from SARS patients “The site of sampling, the exact time after infection and the method used to measure cytokines are therefore critical

determinants with fundamental effects on the interpretation of such studies" (Openshaw, 2004).

APP peaked later than cytokines and, unlike for the cytokines, their levels were higher in serum than in BALF. CRP and HG were mainly increased in serum, and to a lesser extent in BALF. A rise in serum CRP has been previously reported during natural and experimental infection of humans with influenza (Whicher *et al.*, 1985). Similarly, increased serum HG levels have been described after experimental influenza virus infection of horses (Kent and Goodall, 1991). In contrast to CRP and HG, LBP was detected at relatively high levels in the sera of PBS-inoculated control pigs, which has also been described by Sachse *et al.* (2004). Furthermore, the SIV infection did not change serum LBP concentrations but caused a rise in BALF LBP. The profile of both LBP and HG showed some resemblances with those previously observed after experimental infection of CDCD pigs with porcine respiratory coronavirus (PRCV) (Van Gucht *et al.*, 2006). The PRCV-infected pigs also had constant serum LBP levels and an increase in BALF LBP, while serum HG levels peaked later than in SIV-infected pigs. The finding that LBP increases in BALF and not in serum suggests that there may be local production of LBP in the lungs besides diffusion from the circulation. This hypothesis is supported by some in vitro studies (Dentener *et al.*, 2000) in which A549 human epithelial cells produced LBP in response to IL-1, IL-6 or TNF. The exact role of APP in viral infections is not yet clear. Our APP data agree with the viewpoint that APP induction is a non-specific result of circulating cytokines reaching hepatocytes and thus initiating APP production.

In conclusion, influenza viruses appear to induce highly similar profiles of cytokines and inflammatory mediators in pigs and humans. Our study remains merely descriptive and the mouse model is most suitable to unravel the exact role of specific cytokines, because genes for specific cytokines and receptors can be knocked out. On the other hand, pigs are natural influenza virus hosts and the pathogenesis of influenza in pigs is closer to that in humans. Pigs also show anatomic, physiologic and immunologic resemblances to man. For all these reasons, the pig is a valuable animal model to examine the therapeutic role of cytokine antagonists during influenza infections.

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4.2

ROLE OF IFN-α DURING THE ACUTE STAGE OF A SWINE INFLUENZA VIRUS INFECTION

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Abstract

Cytokines, especially interferon-alpha (IFN-α) are important in controlling influenza virus infections. To investigate the role of IFN-α in influenza, the swine IFN-α neutralizing monoclonal antibody (Ab) K9 was applied in a swine model of influenza A virus infection. First, the optimal dose and route for administration of the IFN-α neutralizing Abs was determined. Based on those results, the effect of the Abs on a swine influenza virus infection was investigated. Pigs were inoculated intratracheally with $10^{6.0}$ mean egg infectious dose (EID_{50}) A/Swine/Belgium/1/98 (H1N1) virus. At the time of challenge and 18 hours later, they were injected intratracheally and intraperitoneally with a high dose of IFN-α neutralizing Abs or control Abs. The animals were euthanized at 0, 24, 30, 48 and 72 h after inoculation. At 24 and 30 h, IFN-α levels in broncho-alveolar lavage fluid of K9 recipient animals were strongly suppressed, and this coincided with reduced IL-6 and IL-12 levels. TNF and IL-1 levels were unaffected compared to those in the control Ab treated group. Importantly, the onset and peak of clinical symptoms in IFN-α neutralizing Abs treated animals were delayed by 24 hours, simultaneously with the suppression of IFN-α, but there was no obvious effect on virus replication and lung pathology. These results suggest an important role for IFN-α in IL-6 and IL-12 induction and a role of all 3 cytokines in the symptoms of swine influenza.

Introduction

Interferons represent a class of evolutionary conserved cytokines that have several pathophysiological functions. Most notably, IFNs have potent antiviral activity and they are induced in cells upon virus infection. Virus specific RNA (5' triphosphate moieties of single-stranded RNA and double-stranded RNA) is recognized by the cell as non-self and initiates interferon production (Bowie and Fitzgerald, 2007). The antiviral effect of type I interferon has been extensively studied and works through the induction of antiviral proteins (notably protein kinase R (PKR), 2',5'-oligoadenylate synthetase and Mx protein) interfering with viral replication (Haller *et al.*, 2007). Interferon-α (IFN-α) is also an important mediator of disease symptoms. It induces fever (Dinarello *et al.*, 1984; Kurokawa *et al.*, 1996) and other effects like chills, myalgia, malaise, headache and fatigue in healthy human volunteers and cancer patients treated with IFN (Scott *et al.*, 1981; Gutterman *et al.*, 1982; Corssmit *et al.*, 1995). In mice IFN can induce anorexia (Segall and Crnic, 1990). IFN-α also induces a transient rise in neutrophil counts in the blood at 8 h post IFN-α injection in human volunteers and this effect disappeared at 24 h post injection (Scott *et al.*, 1981; Corssmit *et al.*, 1997). These symptoms also occur in typical influenza infections in humans and swine and IFN-α is thus considered a key mediator of symptoms in influenza (Majde, 2000).

Influenza in man and pigs is characterized by a profuse production of cytokines. In human volunteers inoculated intranasally with influenza, IFN-α, interleukin-6 (IL-6) and tumour necrosis factor (TNF) were demonstrated in nasal lavage fluid. The increases in IFN-α and IL-6 correlated strongly with each other and with symptoms (Hayden *et al.*, 1998). Influenza in pigs can be reproduced by intratracheal (IT) inoculation of influenza-naive pigs with a high dose of swine influenza virus (SIV). This results in the secretion of IFN-α, IL-6, TNF and IL-1 in broncho-alveolar lavage fluid (BALF) (Van Reeth *et al.*, 2002). Similar to humans, IFN-α and IL-6 were the most abundantly produced cytokines following SIV infection and their levels correlated strongly with each other, virus replication in the lungs and disease symptoms (Van Reeth *et al.*, 2002). These results were recently confirmed and extended in a study in which we demonstrated a strong up-regulation of additional

cytokines (IFN-γ and IL-12) and several acute phase proteins during the acute stage of a SIV infection (Barbé *et al.*, 2009).

Current knowledge on the role of IFN-α in the pathophysiology of influenza has been obtained almost entirely from studies in laboratory mice. Much less is known about the role of IFN-α in human or swine influenza infection. In older studies in laboratory mice IFN-α neutralizing antibodies (Abs) were used. Intravenous administration of partially purified sheep anti-mouse interferon globulin in intranasally influenza-inoculated mice did not influence the course of disease (Gresser *et al.*, 1976). In a later study, in contrast, mice infected with influenza virus by aerosol and treated intranasally with a sheep polyclonal anti-interferon serum died within 7 days post inoculation whereas controls survived. Lung virus titres in IFN-α antiserum treated mice were also higher than in controls (Hoshino *et al.*, 1983). These results suggest that interferon plays an important role during the early stage of an influenza virus infection. More recent studies involved mice deficient in IFN-α/β receptor or other proteins involved in the intracellular IFN signalling pathway. Mice lacking the signal transducer and activator of transcription 1 (STAT1) protein, which is activated when type I or type II IFNs bind to their receptors, were 100-fold more sensitive to lethal influenza infection and virus replication was not limited to the respiratory tract (García-Sastre *et al.*, 1998). Nevertheless, other studies in the mouse model of influenza failed to document a protective role of IFNs. Mice deficient in the IFN-α/β receptor were equally susceptible to and had similar lung virus titres than control animals upon challenge with X31 (H3N2) virus (Price *et al.*, 2000). The genetic background of the mice and the virulence characteristics of the challenge strains may in part explain these contradictory findings.

In this study we used an established model in swine for influenza A virus infection and interfered with the type I IFN response by injecting an IFN-α neutralizing monoclonal antibody dose at the onset of infection and 18 hours later. This porcine IFN-α1 neutralizing mouse monoclonal, designated clone K9, was selected from twenty-eight hybridomas and had the highest IFN-α neutralizing capacity (Lefèvre and La Bonnardière, 1986; Lefèvre *et al.*, 1990; L'Haridon *et al.*, 1991). The availability of this anti-porcine IFN-α neutralizing Ab gave us the unique opportunity to investigate the role of IFN-α in swine influenza. Pigs are natural influenza hosts and they represent an excellent model to study the pathogenesis of influenza since

they show anatomical, physiological and immunological similarities to humans (Kuiken and Taubenberger, 2008). We first determined the optimal route and dose of administration of the IFN-α neutralizing Abs in a preliminary study. In a second experiment we studied the effect of IFN-α neutralizing Abs on several parameters (clinical symptoms, lung inflammatory changes, lung virus titres, titres of IFN-α and other cytokines in BALF) in caesarean-derived colostrum-deprived (CDCD) pigs inoculated with 10^6 mean egg infectious dose (EID_{50}) H1N1 SIV.

Materials and Methods

Virus and inoculations

The Swine/Belgium/1/98 (H1N1) SIV strain had been isolated from the lungs of fattening pigs during an acute respiratory disease outbreak. The stock used for inoculation represented the third egg passage. All virus inoculations were performed IT using $10^{6.0}$ EID_{50} in 3 ml phosphate-buffered saline (PBS), by inserting a 20-gauge needle attached to a syringe in the trachea through the skin cranial to the sternum. The Local Ethical Committee of the faculty of Veterinary Medicine, Ghent University approved all animal experiments described in this study.

Antibodies and purification

Neutralizing monoclonal Abs against swine IFN-α derived from hybridoma clone K9 were used in this study and have been described earlier (L'Haridon *et al.*, 1991). To obtain high enough quantities of K9 antibodies, ascites was produced in mice according to standard procedures (Jackson and Fox, 1995). The Federal Public Service for health, Food chain safety and Environment approved this procedure. Following collection, ascitic fluid was clarified by centrifugation (1 h, 10 000*g*, 4°C) and the antibody fraction was precipitated in 50% ammonium sulphate. The pellet fraction was dissolved in 10 mM Tris-HCl pH 8.0 and dialysed against the same buffer before loading on a HiPrep DEAE Sepharose Fast Flow column (GE Healthcare Europe GmbH, Diegem, Belgium) using an FPLC apparatus (Pharmacia Biotech, Uppsala, Sweden). K9 Ab containing fractions were obtained by elution using a NaCl gradient and pooled for further use. Monoclonal isotype-matched (IgG1)

anti-pseudorabies virus (PRV) antibodies, 13D12 (Nauwynck and Pensaert, 1995), were produced and purified in the same way and used as control Abs.

Preliminary experiment to determine the optimal dose and route of administration of IFN-α neutralizing Abs

Four conventional ten-day-old piglets were purchased from a farm and housed in HEPA-filtered isolation units and food and water was provided *ad libitum*. Animals received a supplement of milk powder dissolved in water two times a day.

Three pigs were injected with five million neutralizing units (NU), determined as explained further, of IFN-α neutralizing Abs via the intravenous (IV), intraperitoneal (IP) or IT route. The remaining pig was first injected IV with five million NU K9 Abs and 4 days later with the same dose of the Ab IP. At different time points, blood was taken to determine the IFN-α neutralizing Abs titres in serum and pigs were euthanised at 6 or 18 h after the last administration of Abs. Euthanasia was performed by IV injection of an overdose of sodium pentobarbital 20% (Kela Laboratoria).

After euthanasia, the entire lung was excised and the right lung was immediately lavaged with cold PBS to obtain BALF as described elsewhere (Van Reeth *et al.*, 1999). BALF was separated into cells and cell-free fluids by centrifugation. Cell-free BALF was concentrated 20 times by dialysis against polyethylene glycol, centrifuged and used to determine IFN-α neutralizing Abs by an antiviral bioassay as described below. Peritoneal fluid of one of the IP treated animals was also examined for the presence of K9 IFN-α neutralizing Abs.

IFN-α neutralizing Abs administration in SIV inoculated pigs

Twenty-two 3-week-old CDCD pigs were used. The pigs originated from four sows and were housed in Horsefall-type isolation units with positive-pressure ventilation and fed with ultrahigh-temperature-treated cow's milk supplemented with antibiotics. The animals were divided in 3 groups. Two animals were inoculated IT with PBS and received no Abs (mock-inoculated). Ten animals were inoculated with SIV and received 13D12 isotype-matched control monoclonal Abs (control Abs treated). Ten animals were inoculated with SIV and received IFN-α neutralizing monoclonal Abs (IFN-α neutralizing Abs treated).

Based on the results of the preliminary experiment the IFN-α neutralizing or control Abs were administered at the time of SIV inoculation and again 18 h later. At both time points the Abs were injected IT and IP at a dose of 10 000 000 NU IFN-α neutralizing Abs or an equivalent amount (10 mg) of control Abs.

The piglets were clinically monitored several times per day from the start of the experiment until euthanasia. A clinical score was calculated for each pig. One point was assigned for the presence of each of following signs: abdominal thumping, dyspnoea, coughing, anorexia and depression. A respiration frequency of 60 - 90 respirations per minute added 1 point, and > 90 respirations per minute added 2 points to the clinical score. Individual clinical scores thus ranged from 0 to 7.

Both mock-inoculated pigs were euthanised after 24 h. The pigs from the IFN-α neutralizing and control Abs treated groups were euthanised at 24 and 30 h (n = 3 per group), and 48 and 72 h post inoculation (PI) with SIV (n = 2 per group). Euthanasia was performed as described above.

After euthanasia, the lungs were given a gross lesion score representing an estimate of the total lung surface with consolidation. BALF was collected as described above, cleared of residual virus and cell debris by centrifugation and used to determine the level of IFN-α as well as TNF, IL-1, IL-6 and IL-12. IFN-α was determined using a bioassay described below. TNF, IL-1 and IL-6 were assayed using bioassays as described earlier (Van Reeth *et al.*, 1999; Van Reeth *et al.*, 2002). Briefly, TNF titres were determined in a cytotoxicity test in Porcine Kidney subclone 15 (PK(15)) cells and IL-1 activity was measured in a proliferation assay in D10(N4)M cells. IL-6 was assayed in a proliferation assay using B9 cells. All bioassays were run two or three times and geometric mean titres were calculated. IL-12 was determined with an ELISA (Porcine IL-12/IL-23 p40 ELISA (R&D Systems)). The detection limit of this test was 18 pg/ml. All samples were tested in duplicate and the ELISA was performed as instructed by the manufacturer. Total numbers of BALF cells were counted using a Türk chamber. Cytocentrifuge preparations were stained with Diff-Quik® (Medion Diagnostics) to determine neutrophil numbers. A pool from the left apical, cardiac and diaphragmatic lung lobes was sampled and 20% tissue homogenates were prepared for virus titration in Madin-Darby Canine Kidney cells as described previously (Van Reeth *et al.*, 2002). Virus titres were calculated using the method of Reed and Muench (Reed and Muench, 1938).

Quantification of IFN-α and IFN-α neutralizing antibodies

IFN-α was determined by a bioassay described earlier (Van Reeth *et al.*, 2002). Briefly, twofold dilutions of samples were added to Madin-Darby Bovine Kidney (MDBK) cells in 96-well plates. Following overnight incubation, cells were challenged with vesicular stomatitis virus (VSV) and two days later the antiviral effect of the samples was determined. One unit of IFN-α was defined as the reciprocal of the dilution producing 50% inhibition of cytopathogenic effect (CPE). Samples were tested twice and geometric mean titres were calculated.

IFN-α-neutralizing antibody titres were determined by a test based on the IFN-α bioassay. MDBK cells were seeded in 96-well plates at 25 000 cells per well in 100 µl of Dulbecco's modified Eagle medium with 10% foetal calf serum and antibiotics. Following overnight incubation (37°C, 5% CO_2) cells were confluent. In a separate 96-well plate, a twofold dilutions series of samples was made. Next, 5 units (U) of recombinant porcine IFN-α in 25 µl medium was added to each well. After 2 h incubation at 37°C and 5% CO_2, medium was removed from the plate containing MDBK cells and dilution series were transferred to the cells. Fifty µl of medium was added to each well and after 18 h of incubation (37°C, 5% CO_2) cells in sample and virus control wells were challenged with $10^{4.8}$ $TCID_{50}$ of VSV in 50 µl. Uninfected control wells received 50 µl of medium. Two days later, media were removed, and the percentage of CPE was determined after staining with neutral red and determining the optical density (OD) spectrophotometrically at 492 nm. One NU of IFN-α antibodies was defined as the reciprocal of the calculated dilution with 50% CPE. Therefore, the following formula was used: 2^X (X = exponent of the log 2 dilution with OD just below the cut off value + Q). In which the cut off value is the mean OD of virus control and uninfected control wells and Q = (cut off value – OD value just below the cut off value) / (OD value just above cut off value – OD value just below cut off value). Samples were tested twice and geometric mean titres were calculated.

Statistical analysis

Standard Mann-Whitney tests were used to compare mean clinical scores, virus titres and cytokine titres between control Abs and IFN-α neutralizing Abs treated groups. Spearman rank correlation coefficients (ρ) were calculated to compare

individual IFN-α levels in BALF with other cytokine levels, lung virus titres and clinical scores at euthanasia. $P < 0.05$ was taken as the level of significance.

Results

Preliminary experiment to determine the optimal dose and route of administration of IFN-α neutralizing Abs

Table 1 shows the titres of IFN-α neutralizing Abs in serum, peritoneal fluid and BALF at different time points after K9 treatment. Serum IFN-α neutralizing Abs were undetectable in all pigs before the first Abs administration, but they were still present in the animal that received two K9 injections before the second administration ($10^{4.0}$ NU/ml). All routes of administration, except IT, resulted in high and relatively constant levels of IFN-α neutralizing Abs ($10^{4.3}$ NU/ml) in serum throughout the experiment. IP administration of Abs resulted in up to $10^{3.9}$ NU/ml in peritoneal fluid at euthanasia, 6 h post administration and a persistent level of serum Abs. In the BALF, IFN-α neutralizing Abs were only detectable after IT administration. Administration of IFN-α neutralizing Abs did not result in clinical symptoms, elevated BALF cell levels or neutrophil infiltration in BALF (results not shown).

Effect of IFN-α neutralizing Abs treatment on IFN-α titres in the lungs of SIV inoculated pigs

Figure 1 describes IFN-α levels in BALF at different time points after SIV inoculation. In mock-inoculated pigs no IFN-α was present. In control 13D12-Abs treated pigs, IFN-α was found at all time points throughout the experiment. IFN-α titres peaked at 24 and 30 h PI (21 012 U/ml) and decreased thereafter. By contrast, in IFN-α neutralizing Abs treated pigs IFN-α was completely suppressed or only marginally present at 24 and 30 h PI. At 48 and 72 h PI, mean IFN-α levels were comparable (242 and 3 310 U/ml) to those in control Abs treated pigs (690 and 2 121 U/ml).

Table 1. Titres of IFN-α neutralizing Abs in serum, peritoneal fluid and broncho-alveolar lavage fluid (BALF) after administration of IFN-α neutralizing Abs via different ways

Pig No.	Route of administration	Euthanasia at ... h post Abs administration	IFN-α neutralizing Abs titre (log_{10} NU/ml) in ...								
			Serum at ... h post Abs administration							Peritoneal fluid[1]	BALF**
			0	2	4	6	12	18	24		
1[2]	IV[3]		<1.6	4.4	4.3	4.3	4.2	n.d.[4]	4.2	n.a.[5]	n.a.
	IP[6]	6	4.0	4.0	4.1	3.9	n.a.	n.a.	n.a.	3.9	<1.6
2	IT[7]	6	<1.6	3.0	3.2	3.4	n.a.	n.a.	n.a.	n.d.	4.2
3	IP	18	<1.6	4.1	4.3	4.5	n.d.	4.7	n.a.	n.d.	<1.6
4	IV	18	<1.6	4.3	4.2	4.4	n.d.	4.3	n.a.	n.d.	<1.6

(1) determined at euthanasia, (2) pig no. 1 was first given IFN-α neutralizing Abs IV and 4 days later IP, (3) intravenous, (4) not determined, (5) not applicable, (6) intraperitoneal, (7) intratracheal

Table 2. Evolution of gross lung lesions and inflammatory changes in the lungs after intratracheal inoculation of pigs with swine influenza virus and treatment with control Abs or IFN-α neutralizing Abs.

Group	h PI	n	% consolidation of lung surface ± SD	mean cell numbers in BALF (1) ($x10^6$) ± SD		
				Total	Neutrophil	Mononuclear
mock	0	2	0 ± 0	62 ± 14	0 ± 0.4	62 ± 14
control Abs	24	3	1 ± 1	207 ± 67	30 ± 24	194 ± 59
	30	3	3 ± 2	176 ± 19	70 ± 16	137 ± 19
	48	2	7 ± 4	107 ± 21	1 ± 0	106 ± 21
	72	2	7 ± 4	182 ± 76	1 ± 0.4	181 ± 76
IFN-α neutralizing Abs	24	3	1 ± 1	123 ± 50	26 ± 21	103 ± 43
	30	3	1 ± 1	143 ± 54	61 ± 39	103 ± 46
	48	2	6 ± 1	230 ± 37	29 ± 4	216 ± 42
	72	2	28 ± 5	122 ± 30	2 ± 2	120 ± 29

(1) broncho-alveolar lavage fluid

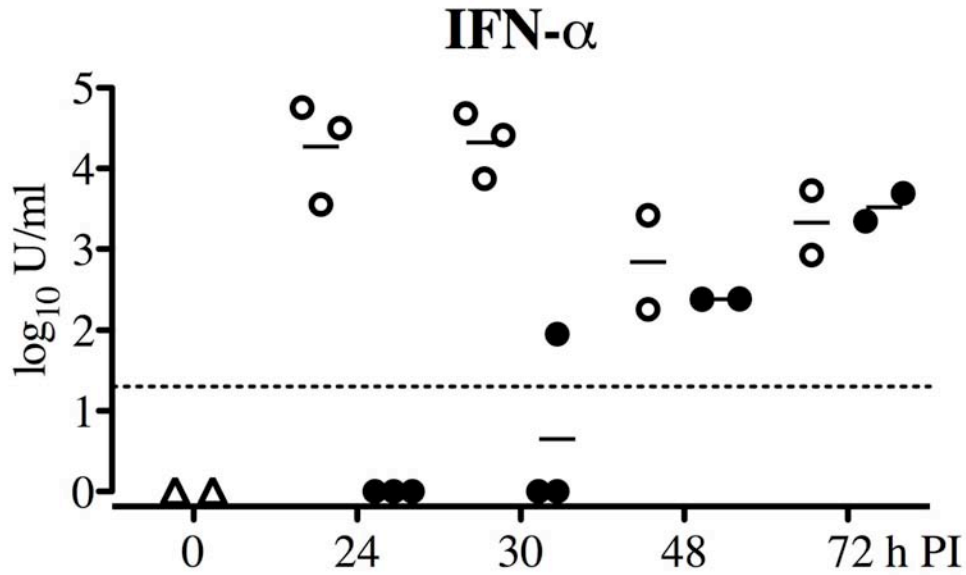

Figure 1. Evolution of IFN-α in broncho-alveolar lavage fluid (BALF) of pigs inoculated with swine influenza virus and treated with IFN-α neutralizing Abs (●) or control Abs (O). Two mock-inoculated control pigs (△) were euthanized at the start of the experiment. Values are expressed as U/ml and geometric means are indicated with a horizontal line. The dotted line is the detection limit of the assay.

Effect of IFN-α neutralizing Abs treatment on clinical symptoms, gross lung lesions and lung inflammatory changes in SIV inoculated pigs

Next we evaluated clinical parameters and lung inflammation in the infected animals. Mock-inoculated animals did not show symptoms, had no lung lesions and displayed only marginal neutrophil infiltration in BALF. Figure 2 shows the evolution of the clinical scores in control Abs and IFN-α neutralizing Abs treated pigs. The control Abs treated animals showed respiratory and general symptoms that started to develop at 21 h PI, peaked at 30 h PI and resolved at 48 h PI. At 30 h PI, 3 out of 4 animals showed symptoms and the mean clinical score was 2.25. Until 24 h PI, IFN-α neutralizing Abs treated animals were free of symptoms. General and respiratory symptoms increased thereafter and peaked at 48 h PI with a mean clinical score of 2.75. At 72 h PI, 1 of the 2 IFN-α neutralizing Abs treated pigs still showed an increased respiration rate (80 respirations per minute) and abdominal thumping. Clinical symptoms were thus delayed compared to control Abs treated pigs.

Table 2 shows gross lung lesions (% consolidation of lung surface), number of cells in BALF, neutrophil and mononuclear cell infiltration in BALF. In control Abs treated animals, consolidation of the lung increased gradually from 1 to 7% throughout the experiment. The number of neutrophils and mononuclear cells in

BALF peaked at 24 and 30 h PI and bordered baseline levels again at 48 and 72 h PI. In IFN-α neutralizing Abs treated pigs consolidation of the lung surface gradually increased from 1% at 24 h PI to 28% at 72 h PI. The number of BALF neutrophils peaked also at 24 and 30 h PI and bordered baseline levels again at 72 h PI. At 48 h PI neutrophil and mononuclear cell levels were slightly higher compared to control animals.

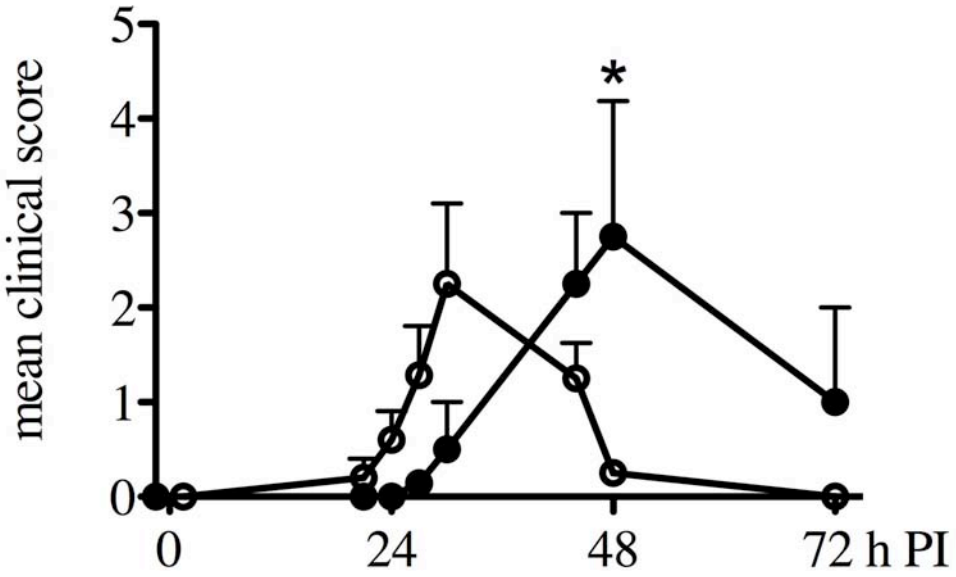

Figure 2. Evolution of clinical scores in pigs inoculated with swine influenza virus and treated with IFN-α neutralizing Abs (●) or control Abs (O). Values are means (+ standard error of the mean). * represents statistically significant difference ($P < 0.05$) between IFN-α neutralizing Abs treated and control Abs treated pigs.

Effect of IFN-α neutralizing Abs treatment on lung virus titres in SIV inoculated pigs

Lungs of mock-inoculated pigs were free of virus (data not shown). Figure 3 describes lung virus titres at different time points after SIV inoculation. In control Abs treated pigs virus titres reached 10^9 mean tissue culture infectious dose ($TCID_{50}$) per gram at 24 and 30 h PI with SIV. At 48 and 72 h PI, when clinical symptoms had subsided, virus titres were about 100 times lower. In IFN-α neutralizing Abs treated pigs, lung virus titres followed the same profile and only differed at 48 h PI when they were $10^{1.8}$ times higher compared to those in control animals.

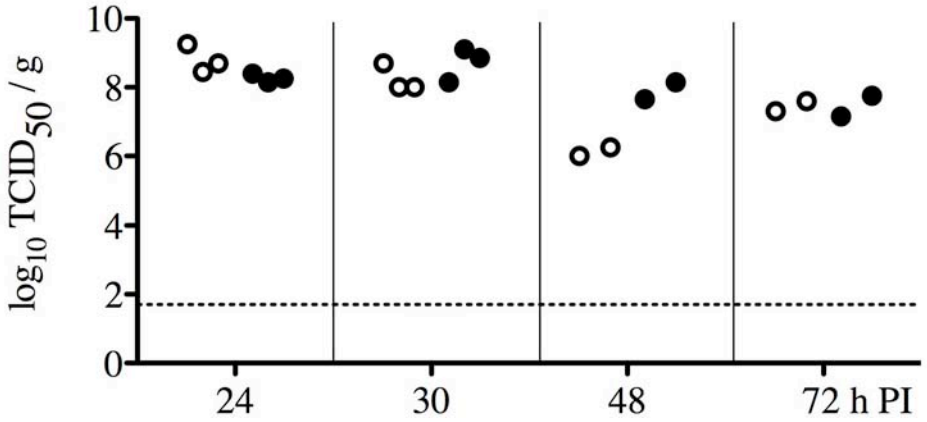

Figure 3. Virus titres in lung tissue homogenates of swine influenza virus inoculated pigs treated with IFN-α neutralizing Abs (●) or control Abs (O). Mock-inoculated pigs were free of virus. The dotted line is the detection limit.

Effect of IFN-α neutralizing Abs treatment on IL-6, TNF, IL-12 and IL-1 titres in BALF of SIV inoculated pigs

IL-6, TNF, IL-12 and IL-1 levels in BALF were determined at different time points PI and are depicted in figure 4. All 4 cytokines were undetectable in mock-inoculated pigs. Control Abs treated pigs showed peak levels of all four cytokines at 24 or 30 h PI. At later time points, these cytokine titres dropped or became undetectable. The IFN-α neutralizing Abs treated pigs displayed increased levels of all four cytokines starting at 24 h PI but levels of IL-6 and IL-12 were lower compared to control Abs treated pigs at 24 h PI, although without statistically significant difference. In addition, peak levels of IL-6 and IL-12 were reached at 48 h PI in IFN-α neutralizing Abs treated pigs. At 48 and 72 h PI cytokine levels were comparable to those in control Abs treated pigs.

Correlations between parameters

Table 3 shows correlations between IFN-α titres and the cytokines measured in BALF, lung virus titres and clinical scores in control or IFN-α neutralizing Abs treated animals. In control animals, IFN-α correlated significantly with IL-6, IL-12, TNF, IL-1 and lung virus titres but not with the clinical scores. In IFN-α neutralizing Abs treated animals IFN-α only correlated significantly with IL-12 and the correlation between IFN-α and IL-6 was not significant ($\rho = 0.23$; $P = 0.53$).

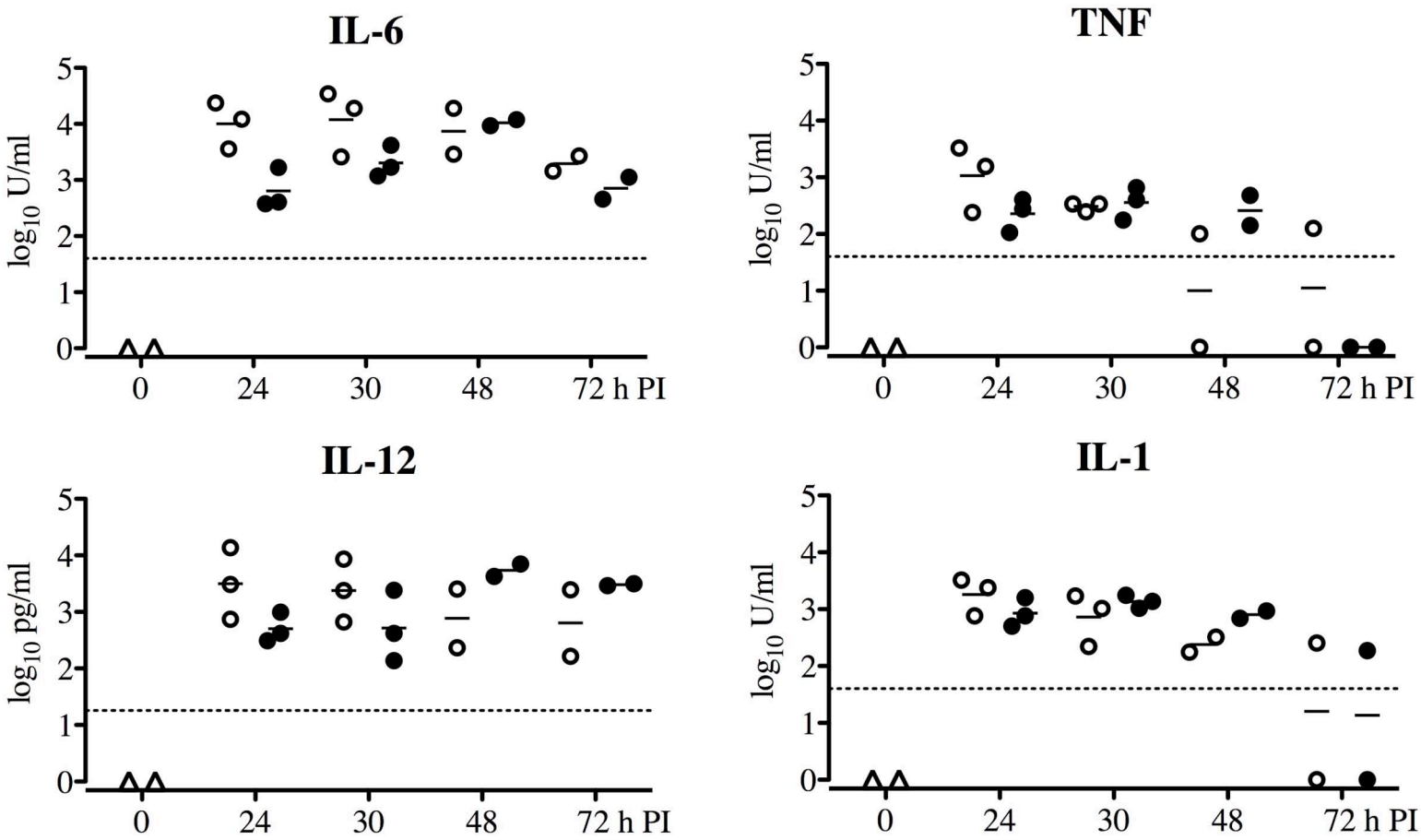

Figure 4. Evolution of IL-6, TNF, IL-12 and IL-1 in broncho-alveolar lavage fluid (BALF) of pigs inoculated with swine influenza virus and treated with IFN-α neutralizing Abs (●) or control Abs (O). Two mock-inoculated control pigs (Δ) were euthanized at the start of the experiment and used as additional controls. Values are expressed as U/ml (IL-6, TNF and IL-1) or pg/ml (IL-12). Geometric (IL-6, TNF and IL-1) or arithmetic (IL-12) means are indicated with a horizontal line. The dotted line is the detection limit of the assay.

Table 3. Spearman rank correlation coefficient (ρ) between IFN-α in broncho-alveolar lavage fluid (BALF) and other cytokines, lung virus titres and clinical scores in control animals or animals treated with IFN-α neutralizing Abs.

IFN-α neutralizing Abs	Correlation of IFN-α with					
	IL-6	IL-12	TNF	IL-1	Virus	Clinical score
-	0.77	0.86	0.94	0.88	0.86	n.s.(1)
+	n.s.	0.81	n.s.	n.s.	n.s.	n.s.

(1) not significant ($P \geq 0.05$)

Discussion

This study aimed to investigate the role of IFN-α in a SIV infection by treatment with porcine IFN-α neutralizing monoclonal Abs. Using this approach we could efficiently suppress IFN-α in BALF during the first 30 h after SIV infection, but IFN-α was again detectable at later time points. Because of the limited number of animals in this study, lung virus titres or BALF cytokine levels did not reach statistically significant differences between control and IFN-α neutralizing Abs treated pigs at specific time points. When data from all animals over the complete period of investigation were cumulated, however, IFN-α and IL-6, but not TNF, IL-1 or IL-12 were significantly lower in IFN-α neutralizing Abs treated animals. Clinical symptoms were clearly delayed in the animals treated with IFN-α neutralizing Abs. This study strongly points towards a role for IFN-α in induction of IL-6, IL-12 and disease symptoms.

The first part of this study focussed on the determination of an optimal route and dose of exogenously administered IFN-α neutralizing Abs. Administration of IFN-α neutralizing Abs via the IP, IT or IV route did not cause any adverse reactions to the pigs and resulted in detectable and long lasting levels of IFN-α neutralizing activity in serum. Intraperitoneal administration seemed to have a depot effect that can be advantageous when IFN-α levels need to be suppressed for an extended period. Intratracheal administration was the only way to reach detectable levels of Abs in BALF, which contains massive amounts of IFN-α in SIV infected pigs (Van Reeth *et al.*, 2002). In contrast with IP or IV administration, IT administration resulted in only

low levels of Abs in the circulation. Therefore, we decided to administer IFN-α neutralizing Abs both via the IP and IT route in the principal experiment.

IL-6 and to a lower extent IL-12 were reduced until 30 h PI in IFN-α neutralizing Abs treated pigs whereas there was no effect on TNF and IL-1. Some of our observations are in line with previous studies. Subcutaneous injection of human volunteers with IFN-α resulted in the induction of IL-6 without effects on TNF and IL-1β levels (Corssmit *et al.*, 1995; Corssmit *et al.*, 1997). These data fit very well with our study where treatment with IFN-α neutralizing Abs reduced IL-6 titres at the early time points after infection. In contrast with our findings, IFN-α appears to inhibit the production of IL-12 in mice in vitro and in vivo. In vitro addition of IFN-α to murine splenic leucocytes reduced the IL-12 inducing capacity of *Staphylococcus aureus* and IFN-α/β neutralizing Abs treated mice had higher levels of IL-12 after inoculation with murine cytomegalovirus (Cousens *et al.*, 1997). Both in the present and in a previous study (Barbé *et al.*, 2009) we found a strong correlation between IFN-α on the one hand and IL-6 and IL-12 on the other, suggesting a biological connection between these cytokines. Difference in IFN-α levels between control and IFN-α neutralizing Abs treated groups are certainly the most likely explanation for the differences in IL-6, IL-12 and clinical symptoms, because there were no differences in virus titres. Other studies have reported that IFN-α is not involved in the induction of IL-1 or TNF, which fits with our study (Corssmit *et al.*, 1995; Corssmit *et al.*, 1997).

The peak of clinical symptoms in IFN-α neutralizing Abs treated pigs was delayed to later time points (from 30 to 48 h PI), as was the detection of IFN-α in BALF. This strongly suggests a role for IFN-α in the induction of disease, reminiscent of the situation in human influenza infections and in human IFN-α recipients, as postulated before by other researchers (Scott *et al.*, 1981; Majde, 2000). Body temperatures were not monitored in the current experiment since this was incompatible with the housing of piglets in sterile conditions. Still, our unpublished preliminary data demonstrated an effect of IFN-α neutralization on the development of fever. It must be taken in account, however, that the effects of IFN-α neutralization on the symptoms of influenza may be indirect through suppression of IL-6 and IL-12.

It is difficult to judge to what extent IFN-α has an antiviral effect in our SIV infection model. The untreated pigs had extremely high lung virus titres at 24 and 30

h PI, together with high IFN-α titres, followed by a drop in virus titres at 48 and 72 h PI. At 48 h PI, the IFN-α neutralizing Abs treated pigs clearly had higher lung virus titres than the controls. Type I IFNs mediate their antiviral effects through the induction of antiviral proteins, which could explain the lack of an antiviral effect during the first 30 h PI. On the other hand, the minimal differences between IFN-α neutralizing and control Abs treated pigs question as to whether IFN-α has significant antiviral activity against SIV under the present experimental conditions. It is known that some influenza viruses can partially escape from the antiviral activity of IFN by the action of their NS1 protein, which binds viral dsRNA that is required for the activation of the IFN-induced antiviral protein PKR (Lu *et al.*, 1995). NS1 deleted swine influenza viruses may be useful to determine whether this may happen during SIV infection.

It is a challenge to neutralize the extremely high amounts of IFN-α produced during a SIV infection by antibody treatment, and to obtain high antibody levels locally in the lungs where IFN-α is produced. These difficulties most likely account for the fact that we could suppress IFN-α only temporarily. Still, the use of IFN-α neutralizing antibodies has allowed us to study the effect of IFN-α during the earliest stage of infection. Knock-out animals for cytokine receptor genes are very difficult to construct in domestic animal species like swine, and we do not consider IFN-α receptor gene knock-out pigs a realistic alternative. Furthermore, the interpretation of the effects of a single cytokine will always be hampered by the redundancy of the cytokine network, whatever approach one uses to block specific cytokines.

In conclusion, exogenously administered IFN-α neutralizing Abs can suppress IFN-α in the pig lung during the very acute stage of a swine influenza virus infection. Although this strategy had a clear effect on IFN-α levels in the lungs, the suppression of IFN-α was only temporal. Despite an obvious delay in the onset of clinical signs, lung virus titres, symptoms and lung pathology were at least as severe as those in the control Abs treated group at 48 and 72 h PI when IFN-α and other cytokines were no longer reduced. Our data indicate a role for IFN-α in IL-6 and IL-12 induction and for a role of all 3 cytokines in the symptoms that are associated with swine influenza.

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5.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

General discussion and future perspectives

Infections with swine influenza virus (SIV) in pigs lead to typical "flu" symptoms and the production of proinflammatory cytokines. This has been well documented before (Van Reeth *et al.*, 1998; Van Reeth *et al.*, 1999; Van Reeth *et al.*, 2002). Despite extensive knowledge about the pathology and disease signs of influenza, the understanding of the disease mechanisms is still incomplete.

The first part of the thesis focussed on the use of commercially available ELISAs as an alternative for the currently used hemagglutination inhibition (HI) test for the detection of SIV specific antibodies (Abs) against European SIVs. A second goal of the research in this thesis was to describe in more detail the response of cytokines and acute phase proteins in SIV inoculated pigs and also to specifically investigate the role of interferon-α (IFN-α) in the pathogenesis of swine influenza. A caesarean-derived colostrum-deprived (CDCD) pig model was used for this aim because this eliminates possible interference with background levels of cytokines due to other infections (Van Reeth *et al.*, 1998).

Three different SIV subtypes are endemic in swine populations worldwide and there are antigenic differences between the same SIV subtypes on different continents (Olsen *et al.*, 2006). With the increasing number of novel subtypes and genetic variants, especially in North America in the last decade, the epidemiology of SIV became more and more complex (Vincent *et al.*, 2008). Consequently, serology for SIV is complicated and may require different antigens in the HI test in different regions of the world. Less work intensive and commercially available techniques such as enzyme-linked immunosorbent assay (ELISA) are available for the detection of antibodies against SIV. Most of these kits are of American origin and have only been validated for the detection against the SIV strains circulating in North America. Because SIV in America and Europe are antigenically different they will induce antibodies that recognize other epitopes. An implication of this fact is that it is not recommended to use ELISAs validated for America in Europe without additional validation. This is especially important for ELISAs based on a whole virus or the hemagglutinin (HA), which have a high antigenic variability, as a coating antigen.

In Chapter 3 of this thesis the IDEXX H1N1 and H3N2 ELISA were validated for the first time with well-defined sera from swine experimentally infected or vaccinated with European SIV isolates. The results of these ELISAs were compared with the

currently used "gold standard" for the detection of antibodies against SIV, the HI test. The latter test is relatively laborious but its advantages are that it is sensitive, subtype and strain specific. The comparison showed that these ELISAs have a low sensitivity and that the ELISAs frequently fail to detect antibodies against European SIVs. It can be expected that other ELISA systems of American origin based on the HA or the whole virus will not perform better. A lesson from these data is that ELISA systems based on the HA of SIV need to be validated separately in different regions of the world. Another option for the detection of antibodies against SIV by ELISA is the use of a species and subtype unrestricted test with the nucleoprotein (NP) of the virus as a coating antigen (Kim *et al.*, 2006). These kits were developed for the detection of avian influenza in poultry. This virus is normally absent in industrially reared poultry and consequently the detection of antibodies would be sufficient for diagnosis of influenza. In contrast, swine influenza is endemic and an at least four-fold rise in NP antibody levels between acute and convalescent serum would be necessary for the diagnosis of a recent SIV infection. To our knowledge, the feasibility of such an approach for diagnosis of swine influenza has not yet been investigated. Another disadvantage is that detection of NP antibodies gives no information about the subtype or antigenic characteristics of the virus. Up to now, an at least four-fold increase in HI titre between acute and convalescent serum in at least one of three separate HI tests with an avian-like H1, a human-like H1 and an H3 is still the method of choice for serological diagnosis of swine influenza.

In Chapter 4.1 we investigated the course of interleukin-12 (IL-12), IFN-γ and IL-18 in a SIV infection. We chose these cytokines because 1) swine specific ELISAs are available for these molecules and 2) IL-12 and IFN-γ are proinflammatory cytokines and IL-18 is supposed to be an IFN-γ inducing factor. Chapter 3.1 indicates that a plethora of cytokines and also acute phase proteins (APP), which were already well investigated in the course of bacterial diseases, are probably involved in the pathogenesis of swine influenza. This hypothesis is based on the fact that there is a very strong correlation between titres of cytokines in broncho-alveolar lavage fluid (BALF) such as IFN-α, tumour necrosis factor (TNF), IL-1, IL-6, IL-12 and IFN-γ with virus replication and disease. Peak levels of these cytokines were found at 24-30 h post inoculation (PI) concurrent with maximal disease symptoms. Levels of these cytokines were much higher in BALF compared to serum. This has been

demonstrated before by other researchers (Hayden *et al.*, 1998) and suggests that cytokines are locally produced at the site of inflammation. Indeed, lung macrophages are very good producers of many cytokines after infection with influenza virus (Cheung *et al.*, 2002). Cytokines might have more local than systemic effects and cytokines present in the circulation after a respiratory infection probably represent only a spill over from the inflamed tissue (Openshaw, 2004), which might be in part due to an increased permeability of the blood vessels in the inflamed tissue.

One of the newly investigated cytokines, namely IL-18, did not follow the classic pattern with a peak at 24-30 h PI of SIV. It was present in BALF and serum of uninoculated control pigs and IL-18 levels decreased after inoculation of SIV and returned to basal levels at the end of the infection. IL-18 is expressed as a precursor molecule intracellularly and the IL-1β converting enzyme (ICE) cleaves it into mature IL-18 and it is afterwards released from the cell. The ELISA used in this research certainly detects the mature form of IL-18 since the standard of the kit is a recombinant mature *Escherichia coli* expressed IL-18 protein. This does not exclude that the kit is also able to detect the pro-IL-18 molecule. A possible way to investigate this is to perform a western blot on BALF, lung tissue and serum samples to check whether the size of the IL-18 molecule matches with the size of the precursor form (24 kDa) or the mature form (20 kDa) of IL-18. Some viruses, such as poxviruses or papillomaviruses, can bind IL-18 and thus they counteract the IL-18 (Xiang and Moss, 1999; Cho *et al.*, 2001). Another possible explanation for the decrease in IL-18 is the induction of an IL-18 binding protein by IFN-α as occurs in chronic hepatitis C patients (Kaser *et al.*, 2002). This possibility can be further investigated by examining the IL-18 levels in the BALF of IFN-α neutralizing Abs treated pigs. Higher IL-18 levels in BALF of such pigs than in non-treated pigs would indicate that IFN-α induces the production of an IL-18 binding protein.

In contrast with cytokines, the role of APP in viral infections is largely unknown. APP are a very heterogeneous group of plasma proteins whose levels rise during inflammatory processes. In this thesis it was also investigated whether APP might play a role in the pathogenesis of an influenza infection. Thereto levels of different APP, namely haptoglobin (HG), lipopolysaccharide (LPS) binding protein (LBP) and C-reactive protein (CRP) in BALF and serum of SIV inoculated pigs were determined. APP levels rose, particularly in serum and less in BALF. APP peaked

later than cytokines, which is an indication that APP are induced by cytokines as is generally accepted (Moshage *et al.*, 1988; Murch, 1998). Higher levels of LBP and HG in serum compared to BALF and a peak of APP that falls later than the cytokine peak was also found earlier in porcine respiratory coronavirus (PRCV) inoculated CDCD piglets (Van Gucht *et al.*, 2006). The role of APP in the pathogenesis of influenza remains unclear. It is however known that increased CRP levels are seen in influenza patients (Falsey *et al.*, 2001; Melbye *et al.*, 2004). It is therefore more logical to postulate that production of APP during a SIV infection is a side effect of the spill over of cytokines from the lungs to the circulation, from where they reach the liver and consequently induce APP as is seen in many bacterial infections (Murch, 1998). For some APP like LBP a local production in lung epithelial cells might also be possible since its levels in BALF rose more than in serum (Dentener *et al.*, 2000).

The above-described correlations are not a proof that these cytokines or APP are involved in the pathogenesis of swine influenza. Thereto cytokine or APP suppressing therapies have to be applied in SIV inoculated animals. The effect of IFN-α in swine influenza was investigated in Chapter 4.2 where porcine IFN-α neutralizing monoclonal antibodies were used to unravel the role of IFN-α in the pathogenesis of swine influenza.

Previous investigations on the effect of proinflammatory mediators in the pathogenesis of porcine respiratory diseases such as porcine reproductive and respiratory syndrome virus (PRRSV) disease involved the cytokine inhibitors pentoxyfilline (a phosphodiesterase inhibitor), and the prostaglandin inhibitors meloxicam and flunixin meglumine (Van Gucht *et al.*, 2004). Pentoxyfilline suppresses TNF production in pigs (Gibson *et al.*, 1991) and the other two molecules are non-steroidal anti-inflammatory drugs that suppress cyclo-oxygenase production and these are registered for use in swine. A porcine model for multifactorial respiratory disease in pigs was used (Van Gucht *et al.*, 2004). In that model the inoculation of PRRSV or LPS, a cell wall component of Gram-negative bacteria, induces only a subclinical to mild disease whereas the subsequent inoculation of PRRSV and LPS has a synergistic effect on symptoms and cytokine production. Only pentoxyfilline reduced BALF levels of the cytokines TNF and IL-1 after dual inoculation with PRRSV and LPS. The two other inhibitors of proinflammatory mediators had no effect on levels of proinflammatory cytokines in BALF after

PRRSV-LPS inoculation. Other investigators also concluded that pentoxyfilline is a moderate inhibitor of proinflammatory mediators in a swine model for acute lung inflammation caused by *Actinobacillus pleuropneumoniae* (Myers *et al.*, 2002). This led to the conclusion that these inhibitors are not adequate tools to study the role of proinflammatory cytokines in the pathogenesis of porcine respiratory diseases because 1) their cytokine suppressing capacity is insufficient and 2) they have a pleiotropic effect. Consequently, more specific cytokine inhibitors, such as antibodies, are needed for such research. These findings inspired us to use monoclonal porcine IFN-α neutralizing antibodies (Abs) to investigate the role of IFN-α in the pathogenesis of a SIV infection. With this approach it was possible to suppress IFN-α during the first 48 hours of a SIV infection. This suppression of IFN-α correlated with a reduction in IL-6 and IL-12 levels and thus IFN-α might be an *in vivo* stimulus for the production of IL-6 and IL-12. This hypothesis can be supported by literature findings. After infection of epithelial cells with influenza virus, these cells produce and release IFN-α. This cytokine triggers leucocytes to produce endogen pyrogens such as IL-1, IL-6, IL-12 and TNF (Brydon *et al.*, 2005). It is thus logical that lower IFN-α levels result in reduced levels of IL-6 and IL-12. The onset and peak of clinical symptoms in SIV inoculated and IFN-α neutralizing Abs treated animals were delayed by 24 h. This finding agrees very well with a mouse model of influenza in which IFN type I receptor knock-out mice had less symptoms early in the infection whereas symptoms were exacerbated later on in the infection (Traynor *et al.*, 2007). In our experimental animal model there was most likely also an effect on the body temperature. Previous unpublished data from our group showed a delayed induction of fever in SIV inoculated and IFN-α neutralizing Abs treated pigs and these animals also had reduced IL-6 levels in BALF. This strongly feeds the hypothesis that IFN-α and IL-6 are strong inducers of fever and disease symptoms occurring in the field after influenza infections in pig herds.

Several studies reported a significant increase in proinflammatory cytokines, a so-called cytokine storm, in severe cases of seasonal influenza, lethal H5N1 and probably also in Mexican flu infections in humans (Peiris *et al.*, 2004; Lee *et al.*, 2007; Gambotto *et al.*, 2008). The syndrome of acute respiratory distress and multiple-organ failure is probably related to this cytokine dysregulation (Beigel *et al.*, 2005). There is scientific interest whether inhibition of the inflammatory cytokine

response might offer a lifesaving therapy for humans. However, mice deficient in the inflammatory cytokines TNF or IL-6 had similar morbidity and mortality as wild-type mice, and it might therefore be preferable to develop therapies that target the virus rather than cytokines in severe cases of influenza (Salomon *et al.*, 2007).

The approach used in the thesis is very promising for the investigation of the role of specific cytokines in the pathogenesis of various diseases. On the other hand, the anti-cytokine strategy is not suitable for treatment of an acute infection such as influenza in swine or humans. The brisk appearance of cytokines, the high cost to produce anti-cytokines antibodies and the redundancy of the effect of different cytokines are reasons that hamper a timely and efficient suppression of the effects of proinflammatory cytokines. Moreover, cytokines are necessary for the development of a good immune response against the invading pathogen. Other cytokine inhibitors that could be used are for example etanercept (Enbrel®), which binds TNF, or anakinra (Kineret®), an IL-1 receptor antagonist. These molecules relieve symptoms in chronic inflammatory processes where there is a relatively mild cytokine production such as in rheumatoid arthritis in humans. Experiments with Enbrel® in PRCV inoculated pigs reduced TNF levels in BALF but had no effect on other cytokines, disease symptoms or virus titres (Atanasova *et al.*, 2009). A combination of different cytokine antagonists in SIV inoculated pigs, for example IFN-α and Enbrel®, might have a bigger effect than the use of only one of the components separately because there is a very tight interplay between cytokines and they stimulate each others production.

IFN-α is a cytokine that links the innate and adaptive immunity (Price *et al.*, 2000; Le Bon *et al.*, 2001; Tough, 2004). IFN-α plays an important role for the transition from an innate to an adaptive immune response (Brassard *et al.*, 2002). This transition to the more efficient and specific adaptive immune response is critical to clear the invading pathogen. Other cytokines such as TNF and IL-12 are also supposed to link the innate and the adaptive immunity (Belardelli and Ferrantini, 2002). Different mechanisms by which IFN-α could modulate the adaptive immune system were described. IFN-α is a differentiation and maturation factor for antigen-presenting dendritic cells and consequently IFN-α is a strong adjuvant for the immune response (Tough, 2004). Subcutaneous injection of IFN-α augments antibody production and induced isotype switching upon antigen stimulation and this indicates that IFN-α

enhances immune responses *in vivo* (Le Bon *et al.*, 2001; Brassard *et al.*, 2002). Besides enhancing the primary response, IFN-α was also able to induce long-term antibody production after a single injection of antigen (Le Bon *et al.*, 2001). It would be interesting to investigate whether IFN-α suppression would have an effect on the specific antibody development and on the cell mediated immune response in SIV inoculated pigs.

As a general conclusion we can state that commercial ELISAs for the detection of antibodies against North American SIVs need to be validated thoroughly before they can be used for diagnosis of swine influenza in other regions of the world. We also created a model for the evaluation of the role of specific cytokines in the pathogenesis of swine influenza and we could prove the pivotal role of IFN-α in the pathogenesis of a SIV infection in an *in vivo* experiment. The experimental animal model used in the present thesis can be used in the future to test the role of other cytokines in the pathogenesis of swine influenza or other porcine respiratory viruses.

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6.

SUMMARY - SAMENVATTING

Summary

Swine influenza viruses (SIVs) of three different subtypes are enzootic in swine producing regions worldwide. Pigs can be used as a valuable animal model for the study of influenza in humans because the disease and pathology show many resemblances with influenza in humans.

In Chapter 1 an introduction to influenza viruses and the pathogenesis of a SIV infection is given. The different options for the serological diagnosis of swine influenza are also summarized. Finally, the introduction summarizes the current knowledge about the cytokine response, with the emphasis on interferon-α (IFN-α), after a swine influenza virus infection.

The aims of the thesis are outlined in Chapter 2. A first major aim was to investigate whether commercially available enzyme-linked immunosorbent assays (ELISA) can be used for the detection of antibodies against European SIVs. The second aim was to investigate in more detail the response of cytokines and acute phase proteins (APP) after a SIV infection, and to study the role of IFN-α in the pathogenesis of swine influenza.

In Chapter 3 it was examined whether a commercial ELISA could serve as an alternative for the currently used gold standard for the serological diagnosis of swine influenza, the hemagglutination inhibition (HI) test. Several ELISAs are on the market. They are often of American origin and since influenza viruses differ antigenically between North America and Europe, a validation is necessary before these tests can be used for the diagnosis of swine influenza in Europe. We tested the capacity of two ELISAs, the IDEXX Swine Influenza Virus H1N1 ELISA and the IDEXX Swine Influenza Virus H3N2 ELISA, to detect antibodies against European SIVs. For this purpose, we used sera from piglets that were singly inoculated with European H1N1, H1N2 or H3N2 SIV; piglets consecutively inoculated with two SIV subtypes; piglets vaccinated with commercial European SIV vaccines; or piglets inoculated with H1N1 and/or H3N2 SIV followed by a single vaccination with a commercial SIV vaccine. These different possibilities mimic the complex

epidemiological situation of swine influenza at the farm level where animals can be infected with one ore more SIV subtypes, where different farms can use different vaccines and where vaccinated animals could have undergone previous infections with SIV. Serum samples of all pigs were collected bi-weekly for 4 to 8 weeks after inoculation or vaccination. All sera were tested by HI tests and IDEXX ELISAs.

Sera of animals vaccinated with commercial SIV vaccines and sera of animals that were experimentally infected with a European H1N1 SIV were often negative in the H1N1 ELISA although they were generally clearly positive in the HI test. Vaccination of animals that had undergone a previous infection with SIV had very high antibody titres in the H1N1 HI test and these animals were also positive in the H1N1 ELISA. The overall relative sensitivity and specificity of the IDEXX Swine Influenza Virus H1N1 ELISA was 39% and 99%, respectively.

The H3N2 ELISA yielded largely the same results. Only a limited number of animals that were positive in the H3N2 HI test were positive in the H3N2 ELISA. Mostly these animals had high HI antibody titres. The overall relative sensitivity and specificity of the IDEXX Swine Influenza Virus H3N2 ELISA was 35% and 97%, respectively.

The data showed that the ELISAs frequently failed to identify pigs infected with European SIVs or vaccinated with commercial European vaccines. Pigs that were positive in the ELISA generally had very high HI antibody titres. The poor sensitivity of the ELISAs may be due to antigenic differences between the SIV strains in the test kits and the strains used for inoculation or vaccination. The HI test thus remains the method of choice for the detection of serum antibodies against SIV although subtype-unrestricted ELISAs based on the conserved influenza virus nucleoprotein (NP) might be an alternative.

Chapter 4 of the thesis extends our knowledge about cytokines and APP in a SIV infection. Chapter 4.1 offers a detailed description of the cytokine and APP response in broncho-alveolar lavage fluid (BALF), serum and lung homogenates of SIV inoculated piglets. To this purpose caesarean-derived colostrum-deprived (CDCD) piglets were intratracheally (IT) inoculated with $10^{7.5}$ EID_{50} Swine/Belgium/1/98 (H1N1) SIV. Piglets were euthanized at 24, 30, 48, 72 and 120 h post inoculation (PI). Two more piglets were mock-inoculated and served as controls. After euthanasia, BALF, serum and lung tissue were collected. The cytokines IFN-α,

interleukin-1 (IL-1), IL-6 and tumour necrosis factor (TNF) were determined by bioassays whereas IFN-γ, IL-12 and IL-18 were determined by ELISAs. Cytokines were determined in BALF, serum and lung tissue. The APP C-reactive protein (CRP), haptoglobin (HG) and lipopolysaccharide binding protein (LBP) were determined by ELISA or colorimetric assay in both BALF and serum. All cytokines, except IL-18, were undetectable in mock-inoculated animals and peaked in all three compartments (BALF, serum and lung tissue) at 24 or 30 h PI. Cytokine levels in BALF were 10 to 100-fold higher than in lung tissue or serum, respectively. At 120 h PI, levels of all cytokines returned to baseline levels. IL-18 levels were lower at 24 and 30 h PI than at 0 h PI and they increased thereafter. The APP CRP and HG peaked 24 h later than the cytokines and reached higher levels in serum than in BALF, while LBP rose in BALF only. Lung virus titres were tightly correlated with BALF IFN-α, IL-6, IL-1, TNF, IFN-γ and IL-12, as well as with serum levels of IL-6 and the interferons. The strongest correlations were found between virus titres and IFN-α and IL-6 in BALF. IL-18 was negatively but not significantly correlated with other parameters. Disease was correlated with the same cytokines in BALF and in serum, as well as with BALF LBP and serum CRP.

We concluded that most investigated cytokines play a role in the pathogenesis of swine influenza. The reason for the aberrant behaviour of IL-18 in a SIV infection needs further investigation. APP appear to be cytokine induced since peak levels of APP are reached at a later time point than peak levels of cytokines. Influenza viruses appear to induce similar profiles of cytokines and inflammatory mediators in pigs and humans. Swine may thus be a valuable experimental animal model for the study of the pathogenesis of influenza.

In Chapter 4.2 the specific role of IFN-α in the pathogenesis of swine influenza was investigated. For this purpose swine IFN-α neutralizing monoclonal antibodies (Abs) were used.

First, the optimal dose and route for administration of the IFN-α neutralizing Abs was determined. For that purpose, conventional piglets were injected with IFN-α neutralizing Abs at a dose of five million neutralizing units (NU) via the intravenous (IV), intraperitoneal (IP) or IT route. At different time points post administration, blood was taken to determine the IFN-α neutralizing Abs titres in serum and pigs

were euthanized at 6 or 18 h after administration of Abs. All routes of administration, except IT, resulted in high and relatively constant levels of Abs in serum throughout the experiment. IP administration of Abs resulted in detectable levels of Abs in peritoneal fluid at euthanasia, and a persistent level of serum Abs. In the BALF, IFN-α neutralizing Abs were only detectable after IT administration.

Next, the effect of IFN-α neutralizing antibodies on the pathogenesis of a SIV infection was investigated. Two CDCD piglets were mock-inoculated and served as controls. Twenty CDCD piglets were inoculated IT with $10^{6.0}$ EID_{50} Swine/Belgium/1/98 (H1N1) SIV. Based on the results of the first part of the experiment, ten pigs were injected IT and IP with 10 million NU IFN-α Abs at the time of virus inoculation and 18 h later. The remaining ten pigs received an irrelevant control Ab. Piglets were euthanized at 24, 30, 48 and 72 h PI. Lung tissue was collected for virus titration and BALF for the determination of cytokines. At 24 and 30 h, IFN-α levels were strongly suppressed in BALF of animals that received IFN-α neutralizing antibodies, and this coincided with reduced IL-6 and IL-12 levels. TNF and IL-1 levels were unaffected compared to those in the control Ab treated group. Importantly, the onset and peak of clinical symptoms in IFN-α neutralizing Abs treated animals were delayed by 24 hours, simultaneously with the suppression of IFN-α, but there was no obvious effect on virus replication and lung pathology.

Intraperitoneally and IT administered IFN-α neutralizing Abs can temporarily suppress IFN-α in the pig lung which is produced during the very acute stage of a swine influenza virus infection. Together with this suppression, there was an obvious delay in the onset of clinical signs, IL-6 and IL-12 in BALF. On the other hand, lung virus titres and lung pathology were at least as severe as those in the control Abs treated group. Our data indicate a role for IFN-α in IL-6 and IL-12 induction and a role for all 3 cytokines in the symptoms that are associated with swine influenza.

In conclusion, we can state that commercial ELISAs for the detection of antibodies against North American SIVs need to be evaluated thoroughly before they can be used for diagnosis of swine influenza in other regions of the world. In this thesis a model for the evaluation of the role of specific cytokines in the pathogenesis of swine influenza was also created. It was also demonstrated that IFN-α plays a paramount role in the pathogenesis of swine influenza. The experimental animal model used in

the present thesis can be further used in the future to test the role of other cytokines in the pathogenesis of swine influenza or other porcine respiratory viruses.

Samenvatting

Drie verschillende subtypes van varkensinfluenza virussen zijn wereldwijd enzoötisch aanwezig in regio's met intensieve varkenshouderij. Varkens kunnen gebruikt worden als een waardevol model voor de studie van influenza bij mensen aangezien het ziekteverloop en de pathologie van varkensinfluenza vele gelijkenissen vertoont met influenza bij de mens.

In Hoofdstuk 1 wordt een inleiding gegeven op influenzavirussen en de pathogenese van een varkensinfluenza virus infectie. De verschillende technieken voor de serologische diagnose van varkensinfluenza worden ook samengevat. Tenslotte vat de inleiding de huidige kennis samen over de cytokine respons, met de nadruk op interferon-α (IFN-α), ten gevolge van een varkensinfluenza virus infectie.

De doelstellingen van de thesis worden vermeld in Hoofdstuk 2. Een eerste belangrijke doelstelling was om na te gaan of commercieel beschikbare enzyme-linked immunosorbent assays (ELISAs) gebruikt kunnen worden voor de detectie van antistoffen tegen Europese varkensinfluenza virussen. De tweede doelstelling was het in meer detail onderzoeken van de respons van cytokines en acute fase eiwitten na een varkensinfluenza virus infectie, en om de rol van IFN-α in de pathogenese van varkensinfluenza na te gaan.

In Hoofdstuk 3 werd onderzocht of een commercieel beschikbare ELISA een alternatief kan vormen voor de huidige gouden standaard voor de serologische diagnose van varkensinfluenza, de hemagglutinatie inhibitie (HI) test. Verschillende ELISAs zijn commercieel beschikbaar. Deze zijn dikwijls van Amerikaanse oorsprong en aangezien influenzavirussen antigenisch verschillen tussen Noord-Amerika en Europa, is een validatie nodig vooraleer deze testen gebruikt kunnen worden voor de diagnose van varkensinfluenza in Europa. We testten de capaciteit van twee ELISAs, de IDEXX Swine Influenza Virus H1N1 ELISA en de IDEXX Swine Influenza Virus H3N2 ELISA, voor het opsporen van antistoffen tegen Europese varkensinfluenza virussen. Hiervoor gebruikten we sera van biggen die enkel werden geïnoculeerd met een Europees H1N1, H1N2 of H3N2

varkensinfluenza virus; biggen die achtereenvolgens werden geïnoculeerd met twee varkensinfluenza subtypes; biggen gevaccineerd met in Europa beschikbare varkensinfluenza virus vaccins; of biggen geïnoculeerd met een H1N1 en/of H3N2 varkensinfluenza virus gevolgd door een enkele vaccinatie met een commercieel varkensinfluenza virus vaccin. Deze verschillende mogelijkheden reflecteren de complexe epidemiologische situatie van varkensinfluenza op bedrijfsniveau, waar dieren kunnen geïnfecteerd zijn met één of meerdere influenzavirus subtypes, waar verschillende bedrijven verschillende vaccins kunnen gebruiken en waar dieren die gevaccineerd worden eerdere infecties met varkensinfluenza virus kunnen hebben ondergaan. Serum stalen van alle varkens werden tweewekelijks verzameld gedurende 4 tot 8 weken na inoculatie of vaccinatie. Alle sera werden getest met HI testen en de IDEXX ELISAs.

Sera van dieren die gevaccineerd werden met commercieel beschikbare varkensinfluenza virus vaccins en sera van dieren die experimenteel geïnfecteerd werden met een Europees H1N1 varkensinfluenza virus waren dikwijls negatief in de H1N1 ELISA. Vaccinatie van dieren die een eerdere infectie met varkensinfluenza virus ondergaan hadden, hadden zeer hoge antistoftiters in de H1N1 HI test en deze dieren waren ook positief in de H1N1 ELISA. De totale relatieve sensitiviteit en specificiteit van de IDEXX Swine Influenza Virus H1N1 ELISA was respectievelijk 39% en 99%.

De H3N2 ELISA gaf over het algemeen hetzelfde beeld. Enkel een beperkt aantal dieren die positief waren in de H3N2 HI test waren positief in de H3N2 ELISA. Meestal hadden deze dieren een hoge HI antistoftiter. De totale relatieve sensitiviteit en specificiteit van de IDEXX Swine Influenza Virus H3N2 ELISA was respectievelijk 35% en 97%.

De resultaten tonen aan dat de ELISAs dikwijls tekort schoten om varkens te detecteren die geïnfecteerd waren met Europese varkensinfluenza virussen of gevaccineerd waren met commerciële Europese vaccins. Varkens die positief waren in de ELISA hadden over het algemeen een zeer hoge HI antistoftiter. De zwakke gevoeligheid van de ELISAs kan te wijten zijn aan antigenische verschillen tussen de varkensinfluenza stammen gebruikt in de ELISA en de stammen gebruikt voor inoculatie of vaccinatie. De HI test blijft dus de voorkeursmethode voor het opsporen van serum antistoffen tegen varkensinfluenza virussen alhoewel niet-subtype

specifieke ELISAs, gebaseerd op het geconserveerde nucleoproteïne van het influenzavirus, mogelijk een alternatief kunnen vormen.

Hoofdstuk 4 van deze thesis handelt over cytokines en acute fase eiwitten tijdens een varkensinfluenza virus infectie. Hoofdstuk 4.1 biedt een gedetailleerde beschrijving van de cytokine en acute fase eiwitten respons in broncho-alveolaire lavage (BAL) vloeistof, serum en long homogenaten van varkensinfluenza virus geïnoculeerde biggen. Hiervoor werden gnotobiotische biggen intratracheaal (IT) geïnoculeerd met $10^{7.5}$ EID_{50} Swine/Belgium/1/98 (H1N1) varkensinfluenza virus. Biggen werden geëuthanaseerd op 24, 30, 48, 72 en 120 h post inoculatie (PI). Twee bijkomende controlebiggen werden geïnoculeerd met fosfaatgebufferde zoutoplossing (FGZ). Na euthanasie werden BAL vloeistof, serum en longweefsel verzameld. De cytokines IFN-α, interleukine-1 (IL-1), IL-6 en tumor necrose factor (TNF) werden bepaald met biologische testen terwijl IFN-γ, IL-12 en IL-18 bepaald werden met ELISAs. Cytokines werden bepaald in BAL vloeistof, serum en longweefsel. De acute fase eiwitten C-reactief proteïne (CRP), haptoglobine (HG) en lipopolysaccharide-bindend proteïne (LBP) werden bepaald met een ELISA of een colorimetrische test in zowel BAL vloeistof als serum. Alle cytokines, met uitzondering van IL-18, waren niet detecteerbaar in controlevarkens en piekten in de drie compartimenten (BAL vloeistof, serum en longweefsel) op 24 en 30 h PI. Cytokine gehaltes in BAL vloeistof waren 10 tot 100 maal hoger dan in respectievelijk longweefsel of serum. De gehaltes van alle cytokines keerden terug naar hun basale niveaus op 120 h PI. De concentratie van IL-18 was lager op 24 en 30 h PI dan op 0 h PI en nam vervolgens terug toe. De acute fase eiwitten CRP en HG piekten 24 h later dan de cytokines en bereikten hogere gehaltes in serum dan in BAL vloeistof, terwijl LBP enkel in BAL vloeistof steeg. Virustiters in de long waren sterk gecorreleerd met IFN-α, IL-6, IL-1, TNF, IFN-γ en IL-12 in BAL vloeistof, en ook met serum gehaltes aan IL-6 en interferonen. De sterkste correlaties werden gevonden tussen virustiters en IFN-α en IL-6 in BAL vloeistof. IL-18 was negatief maar niet significant gecorreleerd met andere parameters. Ziekte was gecorreleerd met dezelfde cytokines in BAL vloeistof en serum als virustiters, en ook met LBP in BAL vloeistof en CRP in serum.

We concludeerden dat de meeste onderzochte cytokines een rol spelen in de pathogenese van varkensinfluenza. De reden voor het afwijkende gedrag van IL-18 in

een varkensinfluenza virus infectie dient verder onderzocht te worden. Acute fase eiwitten lijken geïnduceerd te worden door cytokines aangezien de piek gehaltes aan acute fase eiwitten op een later tijdstip bereikt worden dan deze van de cytokines. Influenzavirussen lijken een zelfde profiel aan cytokines en ontstekingsmediatoren te induceren bij varkens als bij mensen. Varkens kunnen dus een waardevol diermodel zijn voor de studie van de pathogenese van influenza bij de mens.

In Hoofdstuk 4.2 werd specifiek de rol van IFN-α in de pathogenese van varkensinfluenza nagegaan. Hiervoor werden varkens IFN-α neutraliserende monoclonale antistoffen (As) gebruikt.

Ten eerste werd de optimale dosis en toedieningsweg van de IFN-α neutraliserende As bepaald. Hiervoor werden conventionele biggen geïnjecteerd met vijf miljoen neutraliserende eenheden (NE) IFN-α As via de intraveneuze (IV), intraperitoneale (IP) of IT weg. Op verschillende tijdstippen na toediening werd bloed genomen voor het bepalen van de IFN-α neutraliserende As titer in serum, en de varkens werden geëuthanaseerd op 6 of 18 h na toediening van de As. Alle toedieningswegen, met uitzondering van de IT, resulteerden in hoge en relatief constante As gehaltes in serum gedurende het experiment. IP toediening van de As resulteerde in detecteerbare gehaltes van de As in peritoneale vloeistof bij euthanasie, en een persistent gehalte aan serum As. In BAL vloeistof waren IFN-α As enkel detecteerbaar na IT toediening.

Vervolgens werd het effect nagegaan van de toediening van IFN-α neutraliserende As op de pathogenese van een varkensinfluenza virus infectie. Twee gnotobiotische varkens werden als controledieren gebruikt. Twintig andere gnotobiotische varkens werden IT geïnoculeerd met $10^{6.0}$ EID_{50} Swine/Belgium/1/98 (H1N1) varkensinfluenza virus. Gebaseerd op de resultaten van het eerste deel van het experiment werden 10 varkens IT en IP geïnjecteerd met 10 miljoen NE IFN-α As op het moment van virus inoculatie en ook 18 h later. De 10 overblijvende varkens werden geïnjecteerd met een irrelevante controle As. De biggen werden geëuthanaseerd op 24, 30, 48 en 72 h PI. Longweefsel werd verzameld voor virustitratie en BAL vloeistof werd verzameld voor de bepaling van cytokines. Op 24 en 30 h PI was het gehalte aan IFN-α in BAL vloeistof van dieren die IFN-α neutraliserende As kregen sterk onderdrukt, en dit viel samen met verlaagde IL-6 en

IL-12 gehaltes. TNF en IL-1 gehaltes werden niet beïnvloed in vergelijking met deze in de controle As behandelde groep. Een belangrijke observatie was dat het begin en de piek van klinische symptomen bij IFN-α neutraliserende As behandelde dieren werd uitgesteld met 24 h, tezamen met de onderdrukking van IFN-α, maar er was geen duidelijk effect op virusvermeerdering en longpathologie.

Intraperitoneaal en IT toegediende IFN-α neutraliserende As kunnen het IFN-α dat geproduceerd wordt tijdens het hyperacute stadium van een varkensinfluenza virus infectie in de long van varkens tijdelijk onderdrukken. Samen met deze onderdrukking was er een duidelijke vertraging in het opkomen van klinische symptomen en IL-6 en IL-12 in BAL vloeistof. Anderzijds waren virustiters in de long en longpathologie minstens zo uitgesproken als in de controle As behandelde groep. Onze resultaten wijzen er op dat IFN-α een rol speelt in inductie van IL-6 en IL-12 en wijzen op een rol voor deze drie cytokines in het ontwikkelen van de symptomen die gepaard gaan met varkensinfluenza.

Als conclusie kunnen we stellen dat commerciële ELISAs voor het opsporen van antistoffen tegen Noord-Amerikaanse varkensinfluenza virussen grondig moeten gevalideerd worden voor ze kunnen gebruikt worden voor de diagnose van varkensinfluenza in andere regio's van de wereld. In deze thesis werd ook een model ontwikkeld voor het onderzoeken van de rol van specifieke cytokines in de pathogenese van varkensinfluenza. Er werd ook aangetoond dat IFN-α een zeer belangrijke rol speelt in pathogenese van varkensinfluenza. Het diermodel dat gebruikt werd in de huidige thesis kan verder gebruikt worden in de toekomst voor het testen van de rol van andere cytokines in de pathogenese van varkensinfluenza of andere respiratoire virussen.

Curriculum Vitae

PERSONALIA

Filip Barbé werd geboren op 18 augustus 1979 te Ekeren. In 1997 beëindigde hij zijn secundaire opleiding aan het Sint-Eduardusinstituut te Merksem in de richting Wetenschappen-Wiskunde en begon daarna de opleiding Diergeneeskunde te Gent. In 2003 haalde hij het diploma van Dierenarts met onderscheiding aan de Faculteit Diergeneeskunde van de Universiteit Gent. Van oktober 2003 tot augustus 2009 was hij als assistent werkzaam op het Laboratorium voor Virologie, Faculteit Diergeneeskunde, Universiteit Gent. In het kader daarvan hielp hij bij het onderwijs voor de studenten Diergeneeskunde en verrichte een onderzoeksstudie getiteld “Novel insights in the serodiagnosis and pathogenesis of swine influenza” wat geleid heeft tot deze doctoraatsthesis. Sinds september 2009 werkt hij bij Dierengezondheidszorg Vlaanderen VZW als dierenarts diagnosticus voor pluimvee en konijnen. Filip Barbé is auteur van negen wetenschappelijke publicaties, waarvan drie als eerste auteur. Hij nam tevens deel aan verschillende wetenschappelijke congressen in België en in het buitenland.

PUBLICATIONS

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